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## CONTENTS


HOOD, C. L. *The Zoochlorellæ of Frontonia leucas* ..... 79

WHITING, P. W., AND WHITING, ANNA R. *Gynandromorphs and Other Irregular Types in Habrobracon* ..... 89

HUGHES, WINIFRED. *Sex-Intergrades in Fœtal Pigs* ..... 121

WITSCHI, EMIL. *Sex-Reversal in Parabiotic Twins of the American Wood-Frog* ..... 137



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*Vol. LII* *February, 1927* *No. 2*

# BIOLOGICAL BULLETIN

## THE ZOÖCHLORELLÆ OF *FRONTONIA LEUCAS.*

C. L. HOOD,

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### INTRODUCTION.

It is common knowledge that many organisms of the animal kingdom harbor within their bodies various forms of algæ. Dangeard ('02) writes at length on the probable relation of the zoöchlorellæ, in *Paramecium bursaria*, to their host. The experiments of Lipska ('10) on a culture of *Paramecium caudatum* which contained green algæ are also worthy of note. Geza Entz ('81–2) presents evidence which he thinks is indicative of a symbiotic relation between *Stentor polymorphus* and green algæ. Bary ('79) was the first investigator to allude to the relation between the zoöchlorellæ and their host as a symbiotic one. But Dangeard ('02) says that the relation is incompletely proven, and Bouvier adds that we have not a definite example demonstrating the usefulness of the algæ to their host.

The work of this paper was conducted in an effort to add further knowledge on the relation of endoplasmic algæ to their host. The host used in these experiments has been identified as *Frontonia leucas* Ehrenberg.

I wish to express my gratitude to Dr. B. D. Reynolds, under whose direction this work was done, for reading the manuscript and making a number of important suggestions. I am also indebted to Dr. W. A. Kepner and Mr. J. B. Looper for helpful advice and criticisms.

### DISAPPEARANCE OF ZOÖCHLORELLÆ FROM HOST.

In *Frontonia* taken from a collection made near the University of Virginia, from a very small stagnant pool, on June 23, 1925,

the zoöchlorellæ were innumerable. Artificial cultures were attempted with little success. For this reason collections were kept in the laboratory just as they were when taken from the pool, except that fresh spring water was added daily.

After thirty days specimens from these cultures showed a decided decrease in the number of zoöchlorellæ. Fresh collections were then made and treated as before with like results. It was found that with such treatment *Frontonia* would, in from thirty to forty days, be partially if not entirely free of zoöchlorellæ.

On September 15, 1925, seven cultures in fresh spring water [1] were started, using Syracuse watch glasses as containers. Twelve specimens were placed in each culture, and fresh spring water was added daily. By September 18 approximately fifty per

FIG. 1. Camera lucida drawings. × 375. *A*, normal specimen taken on date of collection. *B*, after twelve days in fresh spring water. *C*, after twenty-five days in fresh spring water. *D*, after forty days in fresh spring water.

cent. of the total number of the specimens had disappeared. September 21, all specimens had disappeared from five cultures.

[1] Analysis of the spring water was made by Mr. R. K. Witt, under the direction of Dr. John H. Yoe of the Department of Chemistry, University of Virginia, and showed the following:

| | Parts Per Million. |
|---|---|
| Total solids | 37.6 |
| Fixed residue | 28.0 |
| Loss on ignition | 9.6 |
| Organic matter | Very small amount as indicated by a slight charring on ignition. |

The pH value of the spring water was fairly constant at 7.2.

Three living specimens were found in one, and five in the other. On September 22 duplicate cultures were started with similar results.

September 30, collections of *Frontonia* harboring innumerable zoöchlorellæ were made and change from the original culture medium to fresh spring water was made gradually by drawing off a part of the medium daily and replacing it with fresh spring water. Under these conditions it was observed that within forty days the zoöchlorellæ had completely disappeared, while the ciliates showed no signs of death.

Slides made at intervals extending over the forty-day period corroborate these observations (Fig. 1). Various stains and fixatives were used in making the slides, but the most satisfactory results were obtained from Looper's fixative [1] and iron hæmotoxlyn.

From these observations it is evident that *Frontonia* may be freed from their zoöchlorellæ by a gradual transference from their natural habitat to a medium of fresh spring water.

### Increase in Number of Zoöchlorellæ in Host.

In a collection made from a large semi-stagnant pond, July 2, 1925, *Frontonia* with very few zoöchlorellæ were abundant.

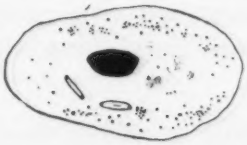

Fig. 2. Typical specimen from a collection in which the *Frontonia* contained but few zoöchlorellæ.

Slides from the collection showed the average specimen to contain from fifty to one hundred zoöchlorellæ (Fig. 2). The

[1] Make a thin smear of Mayer's egg albumen the size of a dime on a clean slide. Place the ciliates to be fixed on the smear in a drop of water just large enough to make a thin layer over the albumen. Invert the slide over the mouth of a small bottle containing a mixture of equal parts of 95 per cent. alcohol, glacial acetic acid and formalin. Let the slide remain in this position 30 seconds to a minute. Remove the slide and slant it back and forth until the film of water has almost disappeared. Wash gently in water and stain.

collection was set aside and remained untouched until August 1. On this date the zoöchlorellæ in twelve specimens examined were innumerable (Fig. 3).

On June 8, 1926, eight cultures, containing twelve specimens per culture, of *Frontonia* harboring relatively few zoöchlorellæ, were started in fresh water to which a small amount of detritis

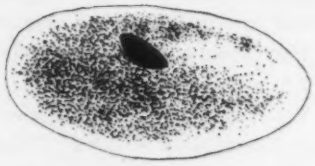

FIG. 3. Showing increase of zoöchlorellæ in *Frontonia* after the same collection (Fig. 2) had remained in the laboratory for thirty days.

from the collecting dish was added. These cultures remained in the laboratory with abundant light, but never in direct sunlight, until July 8, without adding more fresh spring water. At this time slides were made from each culture. The zoöchlorellæ in 95 per cent. of the animals were innumerable. Checks which corroborate these findings were made with cultures started June 10 and 12.

From these results the conclusion, that the number of zoöchlorellæ in *Frontonia leucas* may be increased by an increase in stagnation and putrefaction of the medium in which they live, seems logical.

## INFLUENCE OF OSMOTIC PRESSURE.

In an effort to explain, beyond the simple statement of the difference between stagnant and fresh water, these variations in the number of zoöchlorellæ inhabiting each host, cultures with various osmotic pressures were made as follows: Three cultures using distilled water, three cultures using fresh spring water, three cultures of one per cent. dextrose in spring water. Into each of these cultures were placed six specimens in which the zoöchlorellæ were numerous, and six with no zoöchlorellæ. It was found that upon being put into distilled water the organisms with zoöchlorellæ promptly disintegrated. Ejection of zoö-

chlorellæ, desmids, and diatoms preceding death only by a few moments. The organisms which contained no zoöchlorellæ often lived several hours. In fresh spring water specimens with zoöchlorellæ lived from five to eight days. Specimens without zoöchlorellæ lived indefinitely. In one per cent. dextrose specimens with zoöchlorellæ lived eight days or more. Specimens without zoöchlorellæ averaged two days. It was found also that if the cultures in one per cent. dextrose were placed in fresh media daily, specimens with zoöchlorellæ lived indefinitely, while those without lived four or five days.

Experiments begun June 24, 1926, showed that *Frontonia* with abundant zoöchlorellæ may be induced to live several days in a three per cent. solution of dextrose by beginning at one half per cent., changing the medium daily, and increasing the concentration one half per cent. every fourth day. But under no condition have specimens without zoöchlorellæ been induced to live more than a few hours in a medium exceeding one per cent.

In two cultures containing one per cent. dextrose vegetative reproduction was observed in *Frontonia* harboring zoöchlorellæ where fermentation had been going on three and four days. *Frontonia* without zoöchlorellæ would not live in a medium of this kind.

These observations suggest that specimens harboring zoöchlorellæ are more resistant to media of greater osmotic pressure than are free specimens.

### Hydrogen Ion Concentration.

It has been observed that as fermentation progresses in dextrose cultures the hydrogen ion concentration increases; *e.g.*, a one per cent. solution with a pH value of 7.2 made July 18 showed, 24 hours later, a pH of 7.1. After 48 hours the pH was 7.0, after 72 hours 6.9, and on the fourth day it had become 6.7. Observations made on one, two, and three per cent. solutions showed similar increases in hydrogen ion concentration during fermentation.

The hydrogen ion concentration of media from natural collections was then studied. Data gathered from this study are given in Table I.

## TABLE I.

SHOWING HYDROGEN ION CONCENTRATION OF THREE COLLECTING POOLS OVER AN EXTENDED PERIOD OF TIME.

| Date | August, 1925. | | | | | | | June and July, 1926. | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 4 | 7 | 10 | 13 | 15 | 17 | 12 | 15 | 18 | 21 | 24 | 27 | 30 | 8 |
| *A*... | 7.4 | 7.4 | 7.3 | 7.3 | 7.4 | 7.5 | 7.5 | 7.3 | 7.3 | 7.3 | 7.4 | 7.4 | 7.4 | 7.5 | 7.5 |
| *B*... | 7.4 | 7.3 | 7.3 | 7.3 | 7.4 | 7.4 | 7.4 | 7.3 | 7.3 | 7.2 | 7.2 | 7.3 | 7.3 | 7.4 | 7.4 |
| *C*... | 7.2 | 7.2 | 7.1 | 7.1 | 7.1 | 7.2 | 7.2 | 7.1 | 7.1 | 7.0 | 7.0 | 7.2 | 7.2 | 7.3 | 7.4 |

*A* from pool containing *Frontonia* with no zoöchlorellæ.

*B* with relatively few.

*C* in which the zoöchlorellæ were innumerable.

Beginning June 20th there were increasing rains until July 10. On July 12 collections were taken from all pools where *Frontonia* had previously been found and a uniform scarcity of zoöchlorellæ was observed.

As stated above, when specimens containing relatively few zoöchlorellæ are placed in cultures and left undisturbed, so that the culture medium becomes somewhat stagnant, the zoöchlorellæ increase very noticeably. Also that when specimens containing numerous zoöchlorellæ are changed from their original culture to a medium of fresh spring water, the zoöchlorellæ decrease very noticeably. There is correlated with this increase and decrease in the number of zoöchlorellæ, an increase and decrease in the hydrogen ions. The following examples are characteristic of the finding in this respect.

A collection in which the *Frontonia* showed relatively few zoöchlorellæ had a pH value of 7.3. This culture remained in the laboratory and the hydrogen ion concentration was taken every other day until the specimens harbored innumerable zoöchlorellæ, thirty days. Data thus obtained are given in Table II.

## TABLE II.

SHOWING VARIATIONS IN PH VALUE OF THE MEDIUM AS THE NUMBER OF ZOÖCHLORELLÆ INCREASED.

| | June and July, 1926. | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Date..... | 10 | 12 | 14 | 16 | 18 | 21 | 24 | 27 | 30 | 3 | 6 | 10 | 14 | 18 |
| pH..... | 7.3 | 7.3 | 7.2 | 7.0 | 6.9 | 6.9 | 6.7 | 6.7 | 6.8 | 6.8 | 6.9 | 7.0 | 7.0 | 7.1 |

A collection in which the *Frontonia* showed innumerable zoöchlorellæ had a pH value of 6.8. This culture remained in the laboratory and a change from the original culture medium to fresh spring water was made gradually. The hydrogen ion concentration was taken every other day until the specimens harbored few, and in some cases no zoöchlorellæ. Data thus obtained are given in Table III.

TABLE III.

SHOWING VARIATIONS IN pH VALUE OF THE MEDIUM AS THE NUMBER OF ZOÖCHLORELLÆ DECREASED.

June and July, 1926.

| Date..... | 8 | 10 | 12 | 14 | 16 | 18 | 20 | 22 | 25 | 28 | 30 | 4 | 7 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| pH..... | 6.8 | 6.8 | 6.9 | 6.9 | 6.9 | 7.0 | 7.0 | 7.0 | 7.1 | 7.2 | 7.2 | 7.2 | 7.3 | 7.3 |

## DISCUSSION.

An apparently significant fact observed during the course of these experiments is: That *Frontonia* with abundant zoöchlorellæ refuse to live in media of clear, fresh water. They seem to require a stagnant, even putrid, medium containing detritis, which serves as a place of hiding, much as do *Spirostomum ambiguum*. Indeed these two protozoans have a common habitat in every instance within the experience of the writer. But *Frontonia* without zoöchlorellæ have been found in great abundance in fresh running streams.

It will be noticed from the foregoing investigations that a host may experience either an increase or a decrease in the number of its zoöchlorellæ, but that no statement is made concerning their ability to regain them after having been completely freed. Regarding this subject we may say that repeated efforts to induce *Frontonia* with no zoöchlorellæ to acquire them were unsuccessful. Dangeard says of the zoöchlorellæ of *Paramecium bursaria* that they do not live long when freed in the medium of their host. This suggested the possibility of inducing *Frontonia* without zoöchlorellæ to appropriate them directly from the body of a crushed specimen. Efforts to demonstrate this, however, were unsuccessful.

Another feature that seems worthy of further investigation may be observed in the camera lucida drawings given in Fig. 1; *e.g.*, the evident decrease in the size of the host as the zoöchlorellæ disappear. Minchin quotes Popoff as observing these variations in size of *Frontonia leucas* and crediting them to a difference in temperature of the media in which the *Frontonia* live.

The experiments of this paper were conducted in a laboratory where both large and small, infected and uninfected *Frontonia* were exposed to the same conditions of temperature. Yet differences of as much as 100 microns have been found in specimens taken from the same culture, and these variations have persisted throughout the study. Therefore it seems unlikely that temperature could have had any part in this variation.

Of fifty specimens, without zoöchlorellæ, measured, the average length was 225 microns, while the nucleus averaged 16 microns in length. Of fifty specimens, with innumerable zoöchlorellæ, measured, the average length was 310 microns and the nucleus averaged 18 microns in length.

These observations tend to demonstrate that a mass relation exists between cytoplasm and nucleus. Similar nucleoplasmic relations have been demonstrated in different species of *Arcella* by Hegner (1920) and Reynolds (1923).

Lipska (1910) found that when placed in the dark, *Paramecium caudatum* with zoöchlorellæ would live eight days or more, that *Paramecium* without zoöchlorellæ died between the second and fourth days, and concluded that the zoöchlorellæ were responsible for the greater resistance shown by the *Paramecium* with zoöchlorellæ, in that they furnished a supply of oxygen.

The following experiment was carried out with *Frontonia leucas:* Specimens with zoöchlorellæ were placed in darkness, in their original culture medium, and exposed to the same conditions of temperature as before, but no appreciable difference was noticed in their death rate as compared with those in the light. This, however, does not preclude the possibility of the host obtaining oxygen from the zoöchlorellæ, because in darkness, where photosynthesis is impossible, zoöchlorellæ cannot give off oxygen.

In his work on the zoöchlorellæ of *Paramecium bursaria*, Dangeard found that starch granules were abundant in the zoöchlorellæ and it was his belief that the host appropriated these starch granules as food, though he was unable to demonstrate this.

In this work on the zoöchlorellæ of *Frontonia*, starch granules were found to be abundant in the zoöchlorellæ, but efforts to show that they were ever taken into the food vacuoles of the host have thus far been unsuccessful.

## SUMMARY.

1. *Frontonia* with zoöchlorellæ require a medium of greater stagnation and putrefaction than *Frontonia* without zoöchlorellæ.

2. *Frontonia* may be freed from their zoöchlorellæ by a gradual transference from their natural habitat to a medium of fresh spring water.

3. The number of zoöchlorellæ in *Frontonia* may be increased by increasing the degree of stagnation and putrefaction of the medium in which they live.

4. *Frontonia* harboring zoöchlorellæ are more resistant to media of greater osmotic pressure than are free specimens.

5. The difference between the hydrogen ion concentration of media in which *Frontonia* with innumerable zoöchlorellæ are found and media in which *Frontonia* with no zoöchlorellæ are found, averages 0.3.

6. With an increase in the number of zoöchlorellæ there is a corresponding increase in the hydrogen ions, and conversely.

7. The ability of *Frontonia* harboring zoöchlorellæ to live in a greater concentration of dextrose than *Frontonia* without zoöchlorellæ is correlated with a greater hydrogen ion concentration caused by fermentation of the dextrose.

8. Efforts to induce *Frontonia* without zoöchlorellæ to gain them were unsuccessful.

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# GYNANDROMORPHS AND OTHER IRREGULAR TYPES IN *HABROBRACON*.

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In a series of papers and abstracts on the genetics of the parasitic wasp, *Habrobracon*, mention has been made of various irregular types not falling in line with the general principle of sex-linkoid inheritance.

These cases are considered to be of fundamental significance from the point of view of maturation and cleavage. They include impaternate females, mosaic males and females, gynandromorphs, and intersexual males.

Among the higher Hymenoptera it is now generally assumed that females arise from fertilized eggs, males from unfertilized. Exceptions to this have, however, been claimed in certain ants (Wheeler, W. M., 1903). Among the lower Hymenoptera conditions are much more irregular. Some species of saw-flies are exclusively or predominantly "female-producing," thelytokous; others "male-producing," arrenotokous; while others, amphitokous, produce both sexes parthenogenetically.

Gall wasps with their alternation of sexual and parthenogenetic generations have in the latter both male-producing and female-producing females.

Polyembryonic Chalcidoids produce male broods from unfertilized eggs, female broods from fertilized, but Patterson (Patterson, J. F., 1917) has shown that certain broods, predominantly female, may contain a few males, presumably derived from mitotic irregularities in the polygerm.

Among the Ichneumonoids, to which group *Habrobracon* belongs, arrenotoky is the rule but some species are thelytokous. In a species that is predominantly arrenotokous it has been shown (Hunter, S. J., 1909, and Webster, F. M., 1909) that the tendency to produce females is probably inherited.

The irregular forms discussed in the present paper have

occurred in *Habrobracon juglandis* (Ashmead) with the exception of gynandromorphs, freaks 269 and 271, and intersexual males, freaks 193, 273, 274, and 275. The latter were found in the European species, *Habrobracon brevicornis* (Wesmael).

### Impaternate Females.

In the stock of *Habrobracon juglandis* (Ashmead) from Lancaster, Pennsylvania, females have been, with one very doubtful exception, derived from mated females. In material from Iowa City and in descendents of crosses between the Lancaster (L.) and Iowa City (I.) materials, undoubted cases of thelytoky have occurred.

Females are exposed to males either in culture vial *a* or previously after which they are ordinarily separated. The females are then passed through vials *b*, *c*, etc. Relatively more daughters are produced in the earlier vials. The supply of sperm seems to be exhausted after a length of time varying in individual cases. When female ratios per brood are extremely low the few females usually occur in vial *a*. This applies for example to the daughters of patroclinous diploid males. Single females occurring in later vials may, therefore, be suspected of being impaternate unless other conditions preclude the possibility.

In individual culture counts of the pure I. material, thirty-seven virgin females produced only males, 3,288. Thirty-one females that had been exposed to males produced only males, 3,746. Sixty-three females exposed to males produced both males, 2,933, and females, 2,719. Female percentages in these sixty-three cultures ranged from 18 to 74.

Besides the bisexual broods above recorded there were some in which but a single female appeared.

In one case (April, 1923) a female which was observed to mate with a single male produced 142 males and in vial *c* a single female.

In another case (April, 1923) a female which had been exposed to males produced 97 males and in vial *e* a single female. Each of these two cases is aberrant in that only one female appeared and she was produced relatively late in the mother's life. They are, however, inconclusive as regards thelytoky since the mothers had been exposed to males.

Two cases of undoubted thelytoky occurred in the pure I. material. In one (December, 1922) a virgin female produced 97 males and in vial *b* a single female, freak 277. In the other (April, 1923) a virgin female produced 97 males and in vial *c* a single female, freak 278.

*Freak 276.*—A sooty (mesosternum) female from I. stock 11 ($OD_{II}s_{II}$) was mated to an orange (eye), defective (vein $r_4$) male, $od_{II95}S_{II}$, derived from L. stocks 6 and 10 and I. stock 11. There were produced males—type 1, sooty 49, and females—type 87, grading to sooty 8, and defective 1. One of these type females produced males—type 7, sooty 29, defective 29, sooty defective 4, orange 4, orange sooty 29, orange defective 28, and orange sooty defective 13, showing free segregation of orange and linkage of sooty and defective. In vial *f* an orange female, freak 276, was found (April, 1923). No males appeared. Since the $F_1$ female had been exposed only to her black-eyed brothers her single orange daughter must have been impaternate. She produced males—orange 1, orange sooty 7, orange defective 5, orange defective sooty 8, and females—orange 3, orange sooty 3, orange defective 5. These numbers are not very satisfactory for such variable characters as sooty and defective. Moreover, L. and I. stocks possess minor factors which, when recombined, produce the characters sooty and defective without the factors $s_{II}$ and $d_{II95}$.

It is assumed that thelytoky occurs by the suppression of the second oöcyte division. In this case the first division separated the tetrad into dyad OO which passed into the polar body, and oo which entered the cleavage nucleus.

*Origin of Freaks 250, etc.*—An orange, defective, wrinkled male of the twelfth inbred generation descended from the impaternate female, freak 276, was crossed with a sooty female from I. stock 11. There resulted in $F_1$ 34 black males and 120 black females. Twenty-three of these females were isolated. Fifteen of them had evidently mated with their black-eyed brothers since they produced black-eyed females.

The $F_2$ generation consisted of males—type 217, sooty 240, defective 91, defective sooty 91, wrinkled and wrinkled defective 378, wrinkled sooty and wrinkled sooty defective 123, orange 193,

orange sooty 168, orange defective 133, orange defective sooty 65, orange wrinkled and orange wrinkled defective 390, orange wrinkled sooty and orange wrinkled sooty defective 137, and females—type 900, sooty 16, defective 67, defective sooty 2, besides two impaternate females (freaks 257, 259), one mosaic male (freak 256), and three gynandromorphs (freaks 250, 255, 258) discussed in the present paper.

Disregarding the freaks we find among the total of 2,244 males, 1,086 or 48.39 ± 0.71 per cent. orange, 1,028 or 45.81 ± 0.71 per cent. wrinkled, and 824 or 36.72 ± 0.69 per cent. sooty. Orange is practically equal to expectation. Wrinkled is somewhat below due to death of the most extreme cases in cocoons. Sooty is low on account of method of grading.

Defectiveness was not determined in wrinkled wasps. Among the 1,216 with flat wings there are 398 or 32.73 ± 0.91 per cent. defective. This is lower than expectation when $d_{II95}$ alone is involved although there is always some overlapping with normal. Probably modifiers are involved since linkage between wrinkled, w, and defective, $d_{II95}$, is absent. (Whiting, P. W., 1926*a*.)

Among the 1,216 flat-winged wasps there were 564 or 46.36 ± 0.96 per cent. sooty while among the 1,028 wrinkled there were 260 or 25.29 ± 0.91 per cent. sooty. The relation is contrary to expectation on a basis of linkage. The explanation is found in the fact that wrinkled wasps have lighter mesosterna on the average than flat-winged.

Although we know that factors, o, w; $S_{II}$, and $d_{II95}$ were brought in by the male parent and O, W, $s_{II}$, and $D_{II}$ by the female, no reliance can be placed upon the composition of any $F_2$ individual with respect to $s_{II}$ and $d_{II95}$ without adequate progeny test.

*Freak 257*.—One of the $F_1$ females, $od_{II95}wOD_{II}W$, mentioned under *Origin of freaks 250, etc.*, produced males—type 7, defective 5, wrinkled and wrinkled defective 6, orange 4, orange defective 3, orange wrinkled and orange wrinkled defective 6, and 110 black females besides an orange female (freak 257) with flat wings and normal $r_4$ which appeared in vial *e* (February, 1924). Since no orange females were to be expected we have here a case of thelytoky. Breeding test of this orange female showed her homozygous for normal venation, heterozygous for wrinkled.

Her orange-eyed sons were 35 with flat wings to 44 wrinkled. In 22 of the wrinkled in which $r_4$ could be observed it proved to be normal. The 57 normal demonstrate that $d_{II95}$ was not present and this was evidenced also by later generations.

Freak 257 therefore originated from a first oöcyte division as follows:

$$\frac{\text{First polar body } OOd_{II95}d_{II95}Ww}{\text{Cleavage nucleus } ooD_{II}D_{II}Ww}$$

*Freak 259.*—A second $F_1$ female, $od_{II95}wOD_{II}W$, mentioned under *Origin of freaks 250, etc.*, was set and produced males—type 37, defective 10, wrinkled and wrinkled defective 36, orange 35, orange defective 18, orange wrinkled and orange wrinkled defective 41. In vial *h* an orange female (freak 259) appeared (March, 1924). This female had deformed gonapophyses and sting but was otherwise normal. No offspring could be obtained from it and hence its exact composition as regards $d_{II95}$ and w is not known. Since it had flat wings and normal $r_4$ vein there was present at least one dose of W and probably of $D_{II}$. It demonstrates that for orange the first oöcyte division was reductional.

## Mosaic Males and Females.

Five males with mosaic eyes are reported in the present paper. They are regarded as haploid mosaics from unfertilized eggs (haploid mosaic arrenotoky) in the formation of which both products of the second maturation division (oötids) have become cleavage nuclei. Maturation has been carried a step farther than in the case of impaternate females.

*Freak 65.*—Orange defective from the early L. material was bred up to L. type stock 1 for nine generations alternating with generations segregating orange and defective from the diheterozygous females. An orange defective male of the ninth segregating generation was mated to a type female, L. stock 1. An $F_1$ female produced males—type 11, defective 8, orange 4, orange defective 4, and females—type 2, besides a defective (grade 5) male (freak 65) with orange eyes and black ocelli, appearing in vial *b* (August 4, 1921). Wings and compound eyes of this freak must have been of maternal origin for his

diheterozygous mother, $OoD_{II}d_{II95}$, had mated only with type brothers. Ocelli may have been either of paternal or of maternal origin. The male was absolutely sterile, although very active and willing to mate. It was kept alive forty days and mated to twenty-one females. There were produced no females among 757 males. Dissection showed testes to be unattached. There was a single vas deferens ending blindly on one side, none on the other. Penis and all external parts were normal.

While no evidence exists as to the origin of the black ocelli it may be tentatively assumed by analogy with succeeding cases that this male arose from an unfertilized egg. Oöcyte divisions may be represented as follows:

$$\frac{\text{First polar body} \quad OoD_{II}(D_{II})}{\text{Cleavage nuclei} \quad O(d_{II95}) \mid o(d_{II95})}.$$

As regards orange the first division was equational, the second reductional. We know that $d_{II95}$ remained in the egg for $r_4$ veins were both defective but other tissues might have had either $D_{II}$ or $d_{II95}$. As regards defective therefore, the first division may have been either reductional or equational. Allelomorphs set in parentheses may be exchanged in the formula.

*Freak 256.*—An $F_1$ female, $od_{II95}wOD_{II}W$, of those mentioned under *Origin of freaks 250, etc.*, produced males—type 24, defective 8, wrinkled and wrinkled defective 20, orange 9, orange defective 8, orange wrinkled and orange wrinkled defective 14, and females—type 67, defective 8, and a male (freak 256) with normal $r_4$ vein, flat wings and mosaic eyes appearing in vial *f* (February 19, 1924). Eyes are shown in Figs. 5 and 6. The antennæ of this male had probably been injured by too much ether. It seemed impossible to obtain a mating. Histological preparation showed internal organs apparently normal.

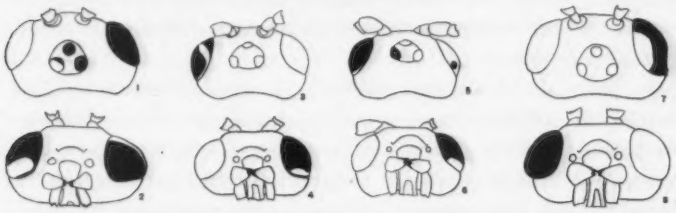
His origin may be represented as follows:

$$\frac{\text{First polar body} \quad Ood_{II95}(d_{II95})w(w)}{\text{Cleavage nuclei} \quad O(D_{II})(W) \mid o(D_{II})(W)}.$$

As regards orange the first division was equational, the second reductional. Because of lack of adequate test, the case for

wrinkled and defective is uncertain. Probably W and $D_{II}$ remained in the egg as evidenced by the flat wings and normal $r_4$ vein of the specimen. In this case the first division may have been either reductional or equational for these factors.

*Freak 154.*—A black female heterozygous for orange, set with two orange brothers from mixed stock (I. x 5) produced males—16 black and 17 orange and females—18 black and 26 orange, besides a male, freak 154, with ocelli and right eye orange, left eye mixed. (February, 1923.) Figs. 3 and 4.



FIGS. 1–8. Dorsal and ventral views of heads of mosaic-eyed males of *Habrobracon juglandis*, showing distribution of black and orange elements. × 29. 1 and 2, freak 185. 3 and 4, freak 154. 5 and 6, freak 256. 7 and 8, freak 264.

Matings with nine orange females resulted in 664 orange males and 598 black females. Only black was therefore transmitted. Maturation of the egg producing this mosaic may be represented as follows:

$$\frac{\text{First polar body } Oo}{\text{Cleavage nuclei } O|o}.$$

Italics are used to denote origin of gonads.

*Freak 185.*—A defective (grade 2) sooty male of mixed ancestry (L. stocks 5, 6, 10, and I. stock 11) arose from a line lacking $d_{II95}$ but having at least one minor factor, $d_x$, causing a small proportion of defectives to appear. This male, $Os_{II}d_x$, was crossed to a female of L. stock 5, $oS_{II}D_x$. A black-eyed virgin daughter produced males—type 45, defective 3, sooty 46, defective sooty 4, orange 43, orange defective 2, orange sooty 42, and in vial *e* a mosaic male, freak 185 (May, 1923).

Eyes and ocelli of this mosaic were mixed black and orange as shown in Figs. 1 and 2. Internal and external genitalia were

normal. Mesosternum was yellow on right side, sooty on left. Sooty is very variable but asymmetry of this trait rarely occurs. Moreover, since the eyes were mosaic for orange and the mother was heterozygous for sooty, this asymmetry may be taken as representing true mosaicism. Vein $r_4$ was absent from the left wing, present in the right. No significance can be attached to this since the trait is commonly asymmetrical.

Freak 185 was crossed to females of orange L. stocks, 3, 5, and 10. There were produced orange males 518, of which 61 graded to sooty, and orange females, 245 or which 23 graded to sooty. These orange females indicate that the mosaic transmitted only orange.

In order to determine whether he transmitted sooty and defective, daughters were tested by breeding. From nineteen daughters of stock 3 ($od_{II95}S_{II}D_x$) females there were produced orange males, 136 of normal venation of which only 30 graded to sooty, and 160 defective of which only 28 graded to sooty. From sixty daughters of stock 5 ($oD_{II}S_{II}D_x$) females there were produced orange males, 1,926 of normal venation of which only 65 graded to sooty and 11 defective with none grading to sooty. The small proportion of $F_2$ males grading to sooty indicates that freak 185 lacks $s_{II}$. The excess of defective over normal from daughters of stock 3 females, and the presence of 11 defective from daughters of stock 5 females indicate that freak 185 transmitted $d_x$.

Mosaic character of eyes and mesosternum indicate that the first oöcyte division giving rise to this freak was equational for orange and probably for sooty. Defective $r_4$ indicates a minor factor for defect remaining in the egg. Breeding tests indicate composition of testes to be $oS_{II}D_x$. Maturation may be represented as follows:

$$\frac{\text{First polar body } OoS_{II}s_{II}D_x(d_x)}{\text{Cleavage nuclei } Os_{II}(D_x)\,|\,oS_{II}d_x}.$$

*Freak 264.*—The orange impaternate female, freak 257, $ooD_{II}D_{II}Ww$, arose from mixed ancestry and probably carried at least one minor factor for defective venation, $d_x$. An orange wrinkled son, $oD_{II}wd_x$, was crossed to a female of L. stock 9

($Od_{II95}WD_x$). There were produced type males 3, defective males 23, and type females 12. One of these females produced males—type 11, defective 22, wrinkled and wrinkled defective 36, orange 11, orange defective 22, orange wrinkled and orange wrinkled defective 28, and in vial *f* a male with mosaic eyes, freak 264 (June 11, 1924). The lack of females indicates that the mother had probably not mated.

Freak 264 had ocelli and left eye orange, right eye black. Figs. 7 and 8. Vein $r_4$ of right wing was normal, of left wing partly defective. The excess of defective over normal brothers indicates at least one minor factor for defect besides the main factor $d_{II95}$.

This mosaic was mated to four females of L. stock 10 ($od_{II95}WD_x$). Male offspring were as expected for stock 10, orange 36, and orange defective 252. Female offspring shown in Table I. were 309 orange and 11 black indicating mosaic character of gonads. From the last column of Table I. it may be seen that percentage of black increased between mating of June 13 A.M. and that of June 13 P.M. (difference $3.16 \pm 1.10$ per cent.) and again between June 13 P.M. and June 14 A.M. (difference $4.18 \pm 2.08$ per cent.). While these differences between successive periods are barely significant, the increase in blacks produced with an increase in age of one complete day is $7.34 \pm 1.66$ per cent. This may indicate that testes were of different character and that the orange-bearing was functioning sooner than the black. We were unfortunately unable to get further matings. Had this been done it is possible that the black daughters might have equaled or exceeded the orange. The black daughters differed moreover in being much more defective than orange. Blacks were $81.82 \pm 7.84$ per cent. defective with mean grade 2.818 while orange were $21.36 \pm 1.57$ per cent. defective with mean grade 0.398.

The eleven black daughters were all tested. They produced males—type 169, defective 188, orange 211, orange defective 221, and females—type 137, defective 156, orange 144, orange defective 141. They were all heterozygous ($D_{II}d_{II95}$) indicating that $d_{II95}$ was in no case received from freak 264. Eighty-seven of the 309 orange daughters produced males—orange 1,359,

## TABLE I.

DAUGHTERS OF MOSAIC-EYED MALE (FREAK 264) ARRANGED ACCORDING TO DATES OF HIS MATINGS.

| Dates of Matings. | Daughters. | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Color. | Normal. | Defective (Graded). | | | | | | | ? | Total. | % Black. |
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | ? | | |
| June 13, A.M. | Orange | 90 | 7 | 15 | 3 | 1 | | | | 1 | 117 | 0.0 |
| June 13, P.M. | Black | 1 | 1 | 1 | | | | | | | 3 | 3.16 ± 1.21 |
| | Orange | 76 | 1 | 7 | 1 | 1 | | | | 6 | 92 | |
| June 14, A.M. (2 matings) | Black | 1 | 1 | 2 | | 1 | 1 | | 2 | | 8 | 7.34 ± 1.69 |
| | Orange | 77 | 6 | 9 | 5 | 3 | | | | 1 | 101 | |
| Total | Black | 2 | 2 | 3 | | 1 | 1 | | 2 | | 11 | 3.44 ± 0.69 |
| | Orange | 243 | 14 | 31 | 9 | 5 | | | | 7 | 309 | |

orange defective 964, and females—orange 1,371, and orange defective 900. There was no correspondence in defectiveness either of black or of orange daughters of freak 264 and defectiveness of their progeny. Defective sons from black females were 51.84 ± 1.20 per cent. and from orange females 41.50 ± 0.70 per cent. The difference is 10.34 ± 1.38 in favor of the former. Defective daughters from black females were 51.38 ± 1.40 per cent. and from orange females 39.63 ± 0.69 per cent. The difference is 11.75 ± 1.56 in favor of the former.

Maturation of the egg producing freak 264 may be represented as follows:

$$\frac{\text{First polar body } \mathrm{Od_{II95}d_{II95}wwD_xd_x}}{\text{Cleavage nuclei } \mathit{OD_{II}Wd_x}\,|\,\mathit{oD_{II}WD_x}}.$$

*Freak 280.*—The mutant male *reduced* (wings), freak 268, occurring in the $F_2$ generation from L. stock 8 female by I. stock 11 male was mated to an L. stock 1 female. A reduced male occurring in $F_2$ from this latter cross, mated to an L. stock 8 female produced 6 type females, no males occurring in the culture. One of these virgin females produced males—type 42, orange 39, reduced 58, orange reduced 45, and a black-eyed male, freak 280, with left primary wing long, right reduced (May, 1925).

An observed mating of this mosaic with an orange-eyed female, stock 8, resulted in orange males 98, and one black female. Unfortunately this female died without offspring and no more progeny could be obtained from the mosaic. The one black-eyed daughter demonstrates that the mosaic transmitted black.

According to the theory advanced for the production of males with mosaic eyes, freak 280 might be formed as follows:

$$\frac{\text{First polar body } \mathrm{o(o)Rr}}{\text{Cleavage nuclei } \mathrm{O(R)\,|\,(O)(r)}}.$$

*Freak 279.*—An ivory (eye) reduced female, stock 20, mated with a type male, stock 11, produced 18 ivory reduced males and 70 type females and in vial *d* a black-eyed female with left primary wing long, right reduced, freak 279 (April, 1926). This female which had evidently mated with an ivory reduced

brother produced males—type 3, reduced 1, ivory 3, and females —type 8, reduced 6, ivory 7, ivory reduced 4. As far as may be judged from these small numbers, this female did not differ in her breeding behavior from her type sisters. The reduced right primary wing may be explained by some local mitotic irregularity involving a single chromosome or a reduction of the whole complex. In the latter case freak 279 may be regarded as a gynandromorph.

## GYNANDROMORPHS.

As a tentative explanation of the origin of gynandromorphs or sex mosaics in *Habrobracon* it is suggested that they may be formed in a manner similar to the mosaic males above discussed, except that one of the oötids is fertilized. There are then two cleavage nuclei, a haploid of maternal origin and a diploid of bisexual origin.

Sex of the various body regions of gynandromorphs is to be recognized by comparison with certain differences between normal males and females. Eyes show no obvious sex difference but sex of the head may be identified in almost all cases by number of joints in antennæ. The long antennæ of the male stand in contrast to the short antennæ of the female. In counts of joints in 2,407 "normal" male antennæ the modal number was twenty-three. A single antenna contained twenty-five while three had only eighteen. In counts of joints in 1,821 "normal" female antennæ the modal number was fifteen; none had over sixteen while four had as low as eleven. Deviation in "normal" individuals is therefore much greater in the minus than in the plus direction. No aberrancies have occurred increasing the number of joints but "deficiency" (Whiting, P. W., 1926*b*) reduces number in either sex. In the latter case antennal joints tend to be fused or tapering. Shortening therefore indicates antennæ as female only if joints are of normal form.

Primary wings of the female average slightly larger than of the male. Variation within each sex is so great that no reliance may be placed on this character. Obvious asymmetry in wing size may, however, be suspected to indicate sex difference between left and right sides of the same gynandromorph.

While some variation obtains in size of sternites, those of the male are much smaller than corresponding sternites of the female. External genitalia of the male bear no resemblance to sting and sensory gonapophyses of the female (Figs. 9–15).

Mating reactions of the male begin with flipping of the wings and running about excitedly when in the presence of the female or if set where a female has recently been. In small vials a male, apparently much excited, may often pass close by a female without noticing her. If, however, his antennæ come in contact with her he will mount and attempt to mate. Copulation is accompanied by a rhythmic beating of wings and antennæ. It may last from three or four seconds to two minutes and may be repeated without dismounting. A male may mate several times in close succession especially if new females are introduced. Males show much individual difference in vigor of mating reactions. The same male may show no reaction at one time and a very vigorous reaction later. Females differ in their capacity to stimulate the response. A male may be stimulated by another male and even mount him but the response is much more pronounced toward a female. A female may remain quiet and permit mating, or give a negative response by running away. If the male persists she kicks him off and bends the tip of her abdomen downward and forward in order to avoid him. He may accomplish a mating in spite of this resistance. A male which is mounted by another male acts in a way somewhat similar but since the aggressor in this case soon desists the negative response is not so pronounced. A female may rarely flip her wings a few times when first introduced to a male. It may be questioned whether this is a sex reaction or a response of a more general type.

Injury to antennæ of males interferes with normal mating reactions. If ether fumes have been too strong there may be an inability to sense the odors from the female. If contact between antennæ of the male and body of the female occurs there may nevertheless be a vigorous response. Antennæ thus injured by ether fragment after two or three days and terminal portions drop off.

Males are entirely indifferent to caterpillars, running over

them hastily as over any other object such as cloth, cotton, paper, etc. Females, on the other hand, have very definite types of reactions towards caterpillars. If a female is touched by a moving caterpillar a defensive reaction occurs. She backs away with abdomen bent downward and forward and with sting extended in a threatening attitude. She then approaches cautiously and if the caterpillar moves draws back again. If the caterpillar remains active she may desist, turn about and move away. A similar threatening or defensive response may be called forth occasionally by other moving objects. If the caterpillar is quiet the female will sting it causing it to squirm violently. The wasp retreats to a safe distance until the caterpillar comes to rest. She approaches again, and again stings the caterpillar which is now unable to react, for its muscles are paralyzed. The reactions of a female wasp toward a paralyzed caterpillar consist in carefully inspecting it with the antennæ, penetrating it with the sting, and feeding from the puncture that she has made.

Oviposition normally follows some time after feeding. Ovipositing reactions begin with antennal inspection. After a favorable spot is selected, usually between the caterpillar and the glass, the abdomen is extended with sting withdrawn and eggs are laid. The wasp may remain in this ovipositing position for many minutes.

Injury to antennæ, unless very extreme, does not prevent normal reactions toward caterpillars.

*Freak 247.*—Along with several males and females reared at room temperature in a vial of stock 13 recently derived from crosses of L. stock 5 and I. stock there was found (January 15, 1924) an orange-eyed gynandromorph with normal wings and male head, twenty-four joints in each antenna. Abdomen was of normal female type except that first right sternite was male. Ovaries and poison sac were normal and seminal receptacle contained sperm.

Tests for reactions extended over four days. The gynandromorph kicked off males attempting to mate, bending its abdomen down slightly to avoid them. It gave positive mating response towards males, flipping wings and running after them but not

mounting. Introduced to females immediately afterwards it gave aggressive mating responses,—flipping of wings, mounting, and rhythmic beating with wings and antennæ. It bent the tip of its abdomen downward in a futile endeavor to copulate. It did not thrust out its sting. Responses towards caterpillars were also like those of a typical male. Contact with an active caterpillar resulted in a simple retreat. No eggs were laid on a paralyzed caterpillar left with it for two days. Freak 247, therefore, acted in every way like a typical male.

*Freak 206.*—From matings between the original I. stock and L. stock 5 a certain female was isolated after having mated with her brothers. Daughters were similarly isolated in five successive generations. A few freak types appeared with extra legs, deficient digestive tract or deformed thorax. The final isolated female produced males—51 normal and 2 with antennal deficiency and females—32 normal, 1 with left mesothoracic leg and wing lacking, 1 with broadened thorax and an unexpanded wing, 1 with right eye lacking, 1 with right wing shrivelled, and 1 with abnormal arrangement of abdominal dorsites, besides a gynandromorph, freak 206, found in vial *b* (May 21, 1923).

Eyes of this gynandromorph were black like those of the parents. The specimen was hungry when found, the abdomen shrunken. It drank honey water and seemed active and vigorous. A preliminary count showed sixteen joints in one antenna which was complete, eighteen in the other which was incomplete and must therefore have had at least nineteen joints originally. Two days later it was noticed that segments were breaking off, probably due to over-etherization. The abdomen was of normal female character throughout. Ovaries, poison sac and glands were normal and seminal receptacle contained sperm.

Careful tests carried on over four days indicated no positive reactions toward females or caterpillars. To the former it appeared as indifferent as a female, to the latter as indifferent as a male. Males were kicked off when they attempted mating.

Since antennal "deficiency" occurred in this strain the shorter antenna may have been a "deficient" male antenna. There was, however, nothing abnormal in the form of the joints so that the head was probably in part male, in part female.

*Freak 144.*—A female of original I. stock crossed with a male from the third generation of I. by L. stock 5 produced besides 77 males and 180 females a gynandromorph, freak 144, found in vial *a* (November 29, 1922). Eyes were black as were those of its parents; head was male, twenty-two joints in each antenna; abdomen was entirely female, poison apparatus present, seminal receptacle devoid of sperm.

Freak 144 is of especial interest from the point of view of its responses. These may be described by days as the tests were made.

November 29. A.M., etherized. P.M., reacted indifferently toward caterpillars. Reacted negatively to male that tried to mate with it, turning abdomen down. Flipped wings and chased male but did not mount. Acted similarly toward female.

November 30. Showed very skillful cleaning reactions of antennæ, wings and legs. Displayed no interest in caterpillars. Flipped wings and mounted female as if to mate but soon desisted.

December 1. Virgin female introduced. Gynandromorph showed no reaction at first. Female inspected caterpillar and stuck its abdomen underneath as if to oviposit. Then gynandromorph rushed up and attempted to mate, sticking abdomen down under caterpillar near that of the female. The latter, disturbed, crawled away. Gynandromorph inspected caterpillar with its antennæ in typical female fashion for a few seconds, then left caterpillar and pursued female aggressively mounting again and again, and bending abdomen down as if to mate. Showed no more interest in caterpillar, walking over it indifferently like a male. A male, when introduced, tried to mate but the gynandromorph kicked him off vigorously, bending abdomen down and forward in typical female fashion. Gynandromorph took no interest in stung caterpillar.

December 2. Gynandromorph showed vigorous negative reaction toward males which attempted to mate. Crawled over males indifferently but flipped wings and mounted female. Showed male indifference toward crawling caterpillar with which it came into contact.

December 3. No eggs were found on caterpillar which had been left with it since November 30. Showed negative reaction

as previously to male. Jabbed its protruded sting into cotton plug again and again as is sometimes done by female. Reacted toward a virgin female like a female toward a caterpillar, thrusting out sting above her and below her and showing no flipping of wings.

December 4. Negative reaction toward male. Indifferent reaction toward caterpillar.

Tests of freak 144 show clearly changes in responses between those characteristic of the male and those characteristic of the female, the male type predominating.

*Freak 249.*—A female from orange defective L. stock 3 was crossed with a male of orange L. stock 7. These stocks were derived early in the history of the L. material and consequently were not closely related. An $F_1$ female after mating with her orange defective brothers produced orange males—6 normal, 2 defective, orange females—12 normal, 8 defective, and an orange gynandromorph with normal wings, freak 249, found in vial *a* (February 8, 1924).

The head of freak 249 was obviously male, having twenty-one joints in each antenna. The abdomen was entirely female both externally and internally. Ovaries, poison sac and glands appeared normal. Seminal receptacle contained sperm, showing that a mating had taken place.

Tests for reactions extended for five days. When introduced to females, the gynandromorph responded by all the typical male reactions. A test with a male resulted in no reaction on the part of either although the male mated immediately with a female introduced shortly afterwards. Reactions toward caterpillars were altogether indifferent.

*Freak 246.*—After nine generations of inbreeding of descendents of the impaternate female, freak 276, two branch lines were bred for three generations separately. One gave rise to an orange-eyed female heterozygous for wrinkled wings; the other to an orange wrinkled male. A cross of these two resulted in orange males, 7 flat and 5 wrinkled; orange females, 4 flat and 6 wrinkled, and an orange wrinkled gynandromorph, freak 246, found in vial *a* (January 4, 1924).

Head was clearly male having twenty-three joints in left

antenna, twenty-four in right. All structures of abdomen were female except first left sternite which was of the male type. The sting was deformed at tip. Ovaries and poison apparatus were normal, seminal receptacle devoid of sperm.

Tests for reactions extended for two days only, but were very thorough. Responses were entirely similar to those of a normal male. There was positive mating reaction toward other males but vigorous response toward females which it mounted several times. When males attempted to mate with it, it acted rather indifferent, turned abdomen down slightly and moved away. This reaction was at each time different from the definite negative reaction characteristic of certain females. No reaction toward active caterpillar could be obtained. Gynandromorph simply moved away indifferently. Even contact of caterpillar with the gonapophyses had no effect. No eggs were laid on paralyzed caterpillar left with it over night.

*Freak 267.*—An ivory-eyed mutant male, freak 281, appearing in $F_2$ from a cross of L. stock 10 female by mosaic male, freak 264, was crossed to an L. stock 10 female. Ivory defective stock 17 was derived from descendents of these. A stock 17 female was crossed to an ivory defective wrinkled male of the tenth generation descended from crosses of L. stocks 9 and 10, and descendents of the impaternate female, freak 257. An ivory defective daughter, heterozygous for wrinkled was crossed to a stock 17 male. Among the flat and wrinkled males and the flat females resulting (numbers not recorded) there appeared (February 18, 1925) an ivory defective (grade 5) flat-winged gynandromorph, freak 267.

Antennæ of this freak were definitely male although the left showed terminal fusion, deficiency, with about eighteen joints visible. The right was normal with twenty-one joints. Abdomen was entirely female externally. Ovaries and poison apparatus were normal, seminal receptacle contained sperm.

Tests for reactions extending over five days showed indifference to caterpillars. Males attempting to mate with it were repulsed many times but responses were male-like, the gynandromorph hurrying to get away rather than bending down abdomen. There was no reaction toward females until the fourth day when

slight flipping occurred. On the fifth day there was aggressive reaction,—mounting, etc.

*Freak 258.*—An $F_1$ female, OoWw, from an orange defective wrinkled male by a sooty female, stock 11 (see *Origin of freak 250, etc.*) produced males—type 20, orange 12, wrinkled 15, orange wrinkled 19, and females—type 39, and a black-eyed gynandromorph, freak 258, found in vial *c* (February 26, 1924).

The head of this gynandromorph was female, fifteen joints in each antenna. Mesosternum was yellow; left wing slightly longer than right. Vein $r_4$ of right wing was normal, of left broken. Abdomen was entirely male with normal external genitalia. Digestive tract and abdominal nerve ganglia were apparently normal, as were the penis, sperm sac, and ducts. No testes could be found, either connected with the ducts or in dorsal anterior part of abdomen, as in the gynandromorph, freak 5.

Tests for reactions may be discussed by days abstracted from the record book.

February 27. Gave flipping reaction in presence of females. Ran over active caterpillars without noticing them. Later gave typical female reaction toward caterpillars inspecting them with antennæ, withdrawing and approaching with abdomen thrust forward.

February 28. Slight flipping in presence of various females, but in general attempted to avoid them. Definite female reaction toward caterpillars as before. Tried to sting cotton plug with tip of abdomen.

February 29. Bumped into females without flipping. Then mounted one with definite reaction as if to mate but ran off very excitedly. Inspected paralyzed caterpillar and later cotton plug very carefully with antennæ.

March 1 to March 4. Left with virgin female. No females resulted among 40 males produced.

March 4. Definite female stinging reactions towards active caterpillars. No reaction toward females. Kicked off males attempting to mate with it.

As a result of these tests we may say that reactions were predominantly female but with weak and occasional male responses.

Asymmetry of wings indicates that thorax was probably in part male, in part female. Both male and female structures therefore possess W. Female parts, the eyes, likewise have O. Since both parents possessed O and W this case gives no clue as to the origin of the characters in the gynandromorph.

*Freak 250.*—An $F_1$ female, OoWw, from an orange defective wrinkled male by a sooty female, stock 11 (see *Origin of freak 250, etc.*) produced males—type 40, orange 46, wrinkled 29, orange wrinkled 43, and females—type 62, and a black-eyed, flat-winged gynandromorph with normal $r_4$ vein and sooty mesosternum found in vial *c* (February 9, 1924). Head was male, antennæ each with twenty-two joints. Abdomen was entirely female. Ovaries and poison sac were normal; seminal receptacle was without sperm.

Tested with females it showed flipping of wings and mounting. It was indifferent to caterpillars but pierced cotton plug with sting. Tests extended for six days but were not entirely satisfactory as ends of antennæ broke off probably from over-etherizing.

As regards method of origin the case is inconclusive for the black eyes of the male head may have been inherited from either parent.

*Freak 255.*—An $F_1$ female, OoWw, from an orange defective wrinkled male by a sooty female, stock 11 (see *Origin of freak 250, etc.*), produced males—type 43, orange 29, wrinkled 29, orange wrinkled 25 and females—type 50, and an orange-eyed gynandromorph, freak 255, found in vial *d* (February 16, 1924).

The head of this gynandromorph was male, twenty-two joints in each antenna, wings were flat, left slightly longer than right, $r_4$ veins complete, mesosternum sooty. The abdomen was entirely female, ovaries and poison apparatus normal, seminal receptacle containing sperm.

No interest was shown in caterpillars. Very definite flipping reaction occurred toward females, but gynandromorph did not mount them. The tests indicate male reactions but are not satisfactory as antennæ lost their terminal joints after two days, due probably to over-etherizing.

The case is of interest from the point of view of method of

origin of the male parts. Orange eyes must have come from the heterozygous mother. The flat character of the right wing, probably male, may have been received from either parent.

As regards orange, maturation and fertilization may be expressed as follows:

| First polar body | | O(O) |
|---|---|---|
| Cleavage nuclei | oötids o | (o) |
| | male pronucleus | O |

*Freak 32.*—A black-eyed female from L. stock 1 was crossed to an orange male from the third segregating generation alternating with three successive crosses to L. stock 1. L. stock only was involved. There were produced black males 14 and females 20 and a black-eyed normal-winged gynandromorph, freak 32, found in vial *e* (April 20, 1921).

Head of freak 32 was male, antennæ with twenty-two joints in left, twenty-one in right. Abdomen was normal female type except that first three segments of left side were male (Figs. 22, 23, 24). Internal structures were not adequately studied. Ovaries containing large eggs were observed.

Tests extending for three days brought forth no reactions towards active or paralyzed caterpillars. Reactions toward males attempting to mate were negative, the abdomen was bent down and the gynandromorph attempted to kick off the males. Nevertheless a male succeeded in accomplishing two copulations of about thirty and fifteen seconds respectively. There was not the slightest reaction towards females. The specimen seemed healthy in every way but acted clumsily in cleaning its wings, gonapophyses, etc.

The black eyes of the male head must be of maternal origin. The mother was homozygous.

| First polar body | | OO |
|---|---|---|
| Cleavage nuclei | oötids O | O |
| | male pronucleus | o |

*Freak 248.*—A black-eyed female from I. stock 11 was crossed with an orange male from L. stock 7. There resulted black males 35, black females 133 and a black-eyed, normal winged gynandromorph, freak 248, found in vial *b* (January 28, 1924).

Head was male, left antenna had twenty-two joints, right antenna was broken but there remained thirteen joints. Abdomen was entirely female, ovaries and poison apparatus were normal and there were no sperm in the seminal receptacle.

Tests extending over five days showed no reaction to caterpillars, definite negative reaction to males attempting to mate, no mating reaction toward males, but aggressive response to females, flipping, mounting, etc.

The formula for the method of origin would be similar to that for freak 32.

*Freak 5.*—An orange female of the third generation from the orange mutant male of L. stock was crossed with a black male of the fourth generation of Mifflintown (M.) stock. There were produced 13 orange males, 15 black females, and a black-eyed gynandromorph, freak 5, found in vial *a* (July 22, 1920).

The head of this gynandromorph was female, fifteen joints in each antenna (Fig. 17). Wings were normal. The abdomen was female in the first three segments and entire right side. The left posterior quarter was distinctly male. There was a full set of male external genitalia including penis, and first and second pairs of claspers. A portion of the sting, much malformed, and the right sensory gonapophysis were present (Figs. 18–21). Internal organs were much confused. The digestive tract appeared normal and ran dorsal to the poison apparatus which was slightly toward the right. Poison duct led from the malformed sac to the base of the sting. Poison glands were approximately normal. Seminal receptacle was normally located and devoid of sperm. Nerve ganglia were well developed. Vasa deferentia ended blindly. Testes were far out of position, lying dorsally in the anterior part of the abdomen. They were large and well-formed with cysts of spermatocytes (?) but no sperm. Ovaries were altogether absent.

Reactions of freak 5 seemed entirely female. It was indifferent to females, seemed willing to mate with males and then turned abdomen down to avoid them. It inspected caterpillars with its antennæ and tried to sting them. It also stuck its abdomen under a paralyzed caterpillar as if attempting to oviposit. The tests were all made in one day after which the specimen was killed.

Freak 5 is of interest genetically as it proves that the gynandromorph comes from a fertilized egg and that female parts show paternal influence.

| | | | |
|---|---|---|---|
| First polar body | | | oo |
| Cleavage nuclei | oötids | o | o |
| | sperm nucleus | | O |

*Habrobracon brevicornis* (Wesmael) which has been introduced into the United States from Europe to parasitize the corn borer may be reared on the Mediterranean flour-moth under conditions standardized for *Habrobracon juglandis*. The two species are somewhat similar. Antennæ of brevicornis are longer, normally ranging from nineteen to twenty-eight joints with a mode at twenty-five in the male, and from fifteen to nineteen joints with a mode at eighteen in the female. Sternites in the male, Fig. 32, are, as in *juglandis*, smaller than in the female, Fig. 25. Mating reactions and responses towards caterpillars are similar, but brevicornis is more excitable and active than *juglandis*. Brevicornis is likewise similar in producing males parthenogenetically. It is probable that sex determination is similar but no mutations have been obtained in *brevicornis* to check this.

*Freak 269.*—On May 28, 1925, Mr. A. M. Cloudman found a gynandromorph of *brevicornis* in vial *c* from a pair also producing 60 males and 46 females. The head was male with twenty-five joints in each antenna. Abdomen was apparently of normal female type, Fig. 25. Seminal receptacle was devoid of sperm, ovaries well-formed, and poison apparatus present.

In tests extending over six days no interest was shown in caterpillars, and there were no reactions towards males. The gynandromorph pierced cotton plug with its sting, bending down abdomen. There was vigorous response toward females, flipping of wings, mounting, beating of antennæ, etc.

*Freak 271.*—In a line of *brevicornis* bred by Mr. Cloudman a culture occurred containing a number of freak types. Besides about 75 normal males and 33 normal females, there were about 8 males with compound eyes reduced, one male with left compound eye missing and antenna twisted, 3 males with "stalked" eyes, 6 females with small eyes, and 5 dead pupæ. Moreover in

vial *a* there was found (July 15, 1925) a gynandromorph, freak 271, and in vial *f* (August, 1925) 3 intersexual males (?), freaks 273, 274, and 275. Vials *f* and *g* also contained 15 males and 9 females. In freak 271 the head was male with twenty-seven joints in left antenna, twenty-six joints in right. Abdomen was female except that some of the sternites were rather small approaching the male condition, Fig. 26. Internal organs were typical female, ovaries, poison sac and glands normal and seminal receptacle devoid of sperm.

Tests extended over five days. The gynandromorph had no interest in caterpillars except that once its abdomen was curved downward toward them. Flipping reaction was shown toward males and vigorous response toward females—flipping, mounting, etc. There was also flipping reaction towards *juglandis* female.

### Intersexual Males.

*Freaks 273, 274, 275.*—The three intersexual males (?), sibs of freak 271, had short antennæ and male genitalia.

Freak 273, with nineteen joints in left antenna, eighteen in right, had typical male genitalia but sclerites were asymmetrical, some of them approaching the female size, Fig. 30. Freak 274 with eighteen joints in left antenna, nineteen in right, likewise had typical male genitalia and asymmetrical sclerites, Fig. 31. Freaks 273 and 274 were found dead. Their abdomens were boiled in sodium hydroxide, the chitin flattened and sternites drawn without foreshortening.

Freak 275, found alive and apparently in good condition, had eighteen joints in each antenna but the left was slightly malformed. Normal male genitalia were present and in addition a small female (?) gonapophysis, Fig. 29. Sternites were irregular, some of them approximating the female. Internal organs were typical in every way for the male.

Tests extending over seven days showed no responses to males, females, or caterpillars.

*Freak 193.*—A *brevicornis* female which had been exposed to her brothers produced besides 91 normal males a male (?) with an appendage similar to a small sensory gonapophysis of a female (Figs. 27 and 28). This individual, freak 193, was

found in vial *b* (May 15, 1923). Sternites were similar to those of a normal male. The head was of normal male type with twenty-seven joints in left antenna, twenty-six in right. Internal organs were in every way typical for the male. No tests of responses were made.

## Discussion.

### *Impaternate Females.*

If in parthenogenesis both oöcyte divisions occur without subsequent nuclear fusion but with somatic doubling, or if the first oöcyte division is entirely reductional and the second is suppressed, homozygous individuals are to be expected.

Impaternate females of *Habrobracon juglandis*, however, may be heterozygous. Thus freak 257, homozygous for $D_{II}$, and, like freaks 276 and 259, for o, was at the same time heterozygous for Ww clearly demonstrating that the first maturation division must have been equational for one locus at least. Had it been reductional for Ww one of the derivatives of the first polar body must have returned to fertilize the female pronucleus in order to produce Ww. In that case, however, we would have Oo and $D_{II}d_{II95}$ unless for them the first oöcyte division had been equational.

Either one of two theories are tenable. For all three loci the first division may be equational, a derivative of the first polar body effecting fertilization or the first division may be equational for some loci, reductional for others, diploidism being effected by suppression of the second oöcyte division.

### *Mosaic Males.*

Since mosaic-eyed male freak 185 had a virgin mother, he arose from an unfertilized egg. Since mosaic-eyed male freak 264 had no sisters, he presumably also arose from an unfertilized egg. As regards the other three males with mosaic eyes orange parts of freaks 65 and 256 and black parts of freak 154 as well as defective $r_4$ veins of freak 65 must have been of maternal origin.

As regards method of origin of these males it is assumed that cleavage of the second oöcyte produced a haploid mosaic (haploid mosaic arrenotoky). It is clear that reduction must have

occurred as recessive characters are visible in the body and breeding tests show that gonads are haploid. In the case of freak 264, the aberrant proportion of black and orange daughters, the significant change in this proportion with advancing maturity, and the greater defectiveness of black daughters as well as their greater capacity to transmit defectiveness, indicate that gonads are mosaic rather than diheterozygous.

It may of course be assumed that any two or more of the four products of maturation took part in cleavage. If a derivative of the "first polar body" as well as the "female pronucleus" were thus involved the first division may have been either entirely reductional, entirely equational, or in part reductional, in part equational.

According to the hypothesis assumed, that the first polar body takes no part in embryo formation, the first oöcyte division must be in part reductional, in part equational.

Thus for freaks 65, 154, and 256 Oo must have undergone post-reduction; for freak 185, Oo and probably $S_{II}s_{II}$ underwent post-reduction; for freak 264, Oo and $D_xd_x$ underwent post-reduction, while $D_{II}d_{II95}$ and Ww underwent pre-reduction. In the case of freak 280 the black-eyed male mosaic for reduced, Oo, probably underwent pre-reduction, while Rr underwent post-reduction.

*Gynandromorphs.*

The fourteen gynandromorphs discussed in the present paper came from bisexual fraternities. This is consistent with the theory that they arose from fertilized eggs. Freak 5, with black eyes, female head, gives critical evidence of this for its mother was orange, its father black. It also precludes the possibility of matroclinous female parts developed thelytokously. Three parents of the two gynandromorphs of *brevicornis* and of five of *juglandis* had no obvious factorial differences other than sex. The character shown by three others was possessed by both parents, the mothers being heterozygous.

Freaks 32 and 248 with male head, black eyes, had black mother and orange father, while freak 255 with male head, orange eyes, had heterozygous mother, black father. Male parts of these three must therefore have been derived from the mother. In the case of freak 255 at least reduction occurred.

The evidence does not prove although it is consistent with the theory that male parts are from one oötid, female parts from a fusion of another oötid and the male pronucleus.

*Intersexes.*

The fundamental distinction between gynandromorphs and intersexes is that the former are genetic mosaics while in the latter all parts of the body are presumed to be of similar genetic constitution. Male and female parts of gynandromorphs occur in distinct regions while intersexes are either male with a greater or less female tendency or female with a greater or less male tendency.

Intersex, freak 193, occurred in a male brood, hence in all probability from an unfertilized egg. It is therefore to be considered as an intersexual male.

Intersexes, freaks 273, 274, 275, occurring in one culture, differ from a gynandromorph in that the male and female regions are not definitely defined. They are considered to be male intersexes although their short antennæ may indicate that they are gynandromorphs with female heads.

Freak 271, a sib to freaks 273, 274, and 275, with male head, male responses and female abdomen should be regarded as a gynandromorph. Its irregular sternites and relationship to intersexual males may indicate that it is also intersexual.

*Behavior of Gynandromorphs.*

Responses of mosaic males and of impaternate females do not differ from responses of normal males and females respectively.

The fourteen gynandromorphs and one intersex of *Habrobracon* tested may be grouped with respect to responses as follows:

(1) Typically male. *Juglandis* freaks 246, 247, 248, 249, and 267 were definitely and strongly male. *Juglandis* freak 255 was definitely male but failed to mount females. This failure may be due to injury of antennæ by ether. *Juglandis* freak 250 and brevicornis freak 269 were strongly male but each of these was seen to pierce the cotton plug with its sting, a reaction characteristic of females. The meaning of this reaction is not clear as it is not known what males would do if they had stings. *Brevi-*

*cornis* freak 271 was definitely and strongly male, although it was once observed to direct its sting toward a caterpillar. These nine gynandromorphs had long male antennæ. Abdomens were typically female except for one anterior sternite in 246 and 247, and some irregular reduction of anterior sternites in 271 (Fig. 26).

(2) Predominantly male. *Juglandis* freak 144, although showing definite and vigorous responses toward females, and for the most part no interest in caterpillars, had a definite but very brief female response toward the latter. Moreover it showed decided negative reaction toward males attempting to mate with it and inspected and pierced the cotton plug in a way characteristic of females. It had male antennæ and female abdomen.

(3) Predominantly female. *Juglandis* freak 258 was predominantly female, reacting toward caterpillars and piercing cotton plug. It seemed in general less interested in caterpillars than a normal female and frequently gave flipping reactions toward females, at one time mounting and attempting to mate. It had female antennæ, male abdomen but no testes.

(4) Typically female. *Juglandis* freak 5 acted in every way like a typical female. It had a female head, but a mixed abdomen. Lack of ovaries and presence of poison apparatus and testes are to be noted.

(5) Indifferent. Complete indifference of *juglandis* freak 206 may possibly be ascribed to overdose of ether but this is unlikely. Its female abdomen, one long and one short antenna, might be supposed to cause conflict of tendencies resulting in failure of response. *Juglandis* freak 32 was perfectly healthy but completely indifferent. It had head and first three left abdominal sternites male (Figs. 22, 23, 24). It is possible that had it been kept alive longer than three days it might have reacted toward females, although others gave aggressive responses when much younger. The indifference of *brevicornis* freak 275, intersexual male with short antennæ, mixed abdomen and normal male internal organs, cannot now be explained.

Disregarding the indifferent groups (5) as being not satisfactorily explained as yet we may say that responses are controlled by the head but that there are conflicting tendencies in certain instances.

Presence of ovaries in group 1 and group 2, lack of gonads in group 3, and presence of testes in group 4, show failure of gonads to determine type of response.

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EXPLANATION OF PLATE I.

FIGS. 9–24. Camera lucida drawings of various parts of gynandromorphs of *Habrobracon juglandis*, freaks 5 and 32, and of normal males and females for comparison.

FIGS. 9, 10, 11. Dorsal, sinistral, and ventral view of tip of abdomen of normal male. × 50.

FIGS. 12, 13, 14. Postero-sinistral, sinistral, postero-ventral views of tip of abdomen of normal female. × 50.

FIG. 15. Posterior view of tip of abdomen of normal male. × 50.

FIG. 16. Sinistral view of abdomen of normal male. × 22.

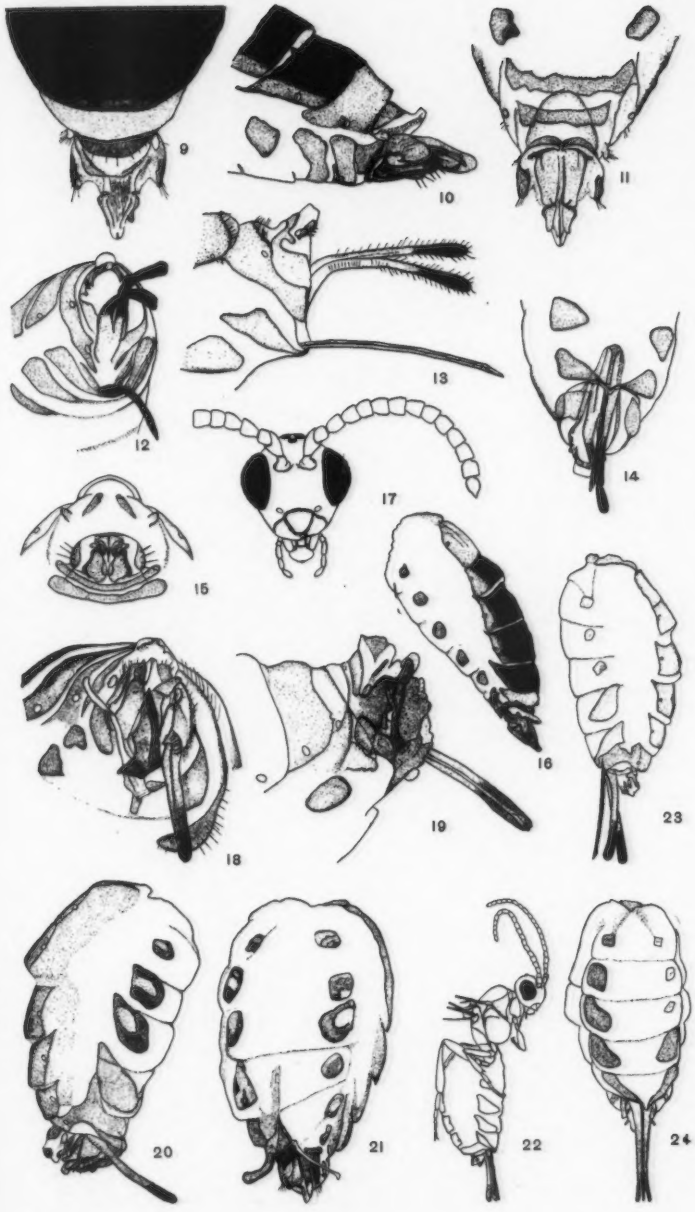
FIG. 17. Anterior view of head of freak 5. × 32.

FIGS. 18 AND 19. Postero-sinistral and sinistral views of tip of abdomen of freak 5. × 50.

FIGS. 20 AND 21. Dextral and ventro-sinistral views of abdomen of freak 5. × 32.

FIG. 22. Dextral view of freak 32. × 9.

FIGS. 23 AND 24. Sinistral and ventral views of abdomen of freak 32. × 17.

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## Explanation of Plate II.

Figs. 25–32. Abdominal sternites and external genitalia of gynandromorphs and intersexes (?) of *Habrobracon brevicornis*. × 29.

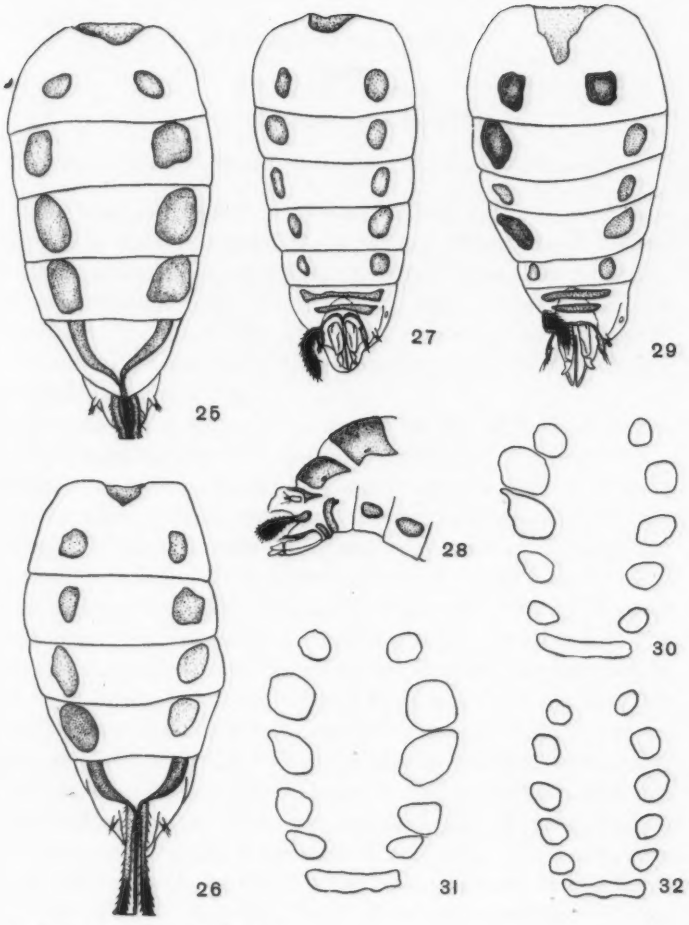
Figs. 25 and 26. Ventral views of abdomens of gynandromorphs, freaks 269 and 271. Fig. 25 is typical for the female.

Figs. 27 and 28. Ventral view of abdomen and dextral view of tip of abdomen of intersexual male (?), freak 193.

Fig. 29. Ventral view of abdomen of intersexual male (?), freak 275.

Figs. 30 and 31. Abdominal sternites of intersexual males (?), freaks 273 and 274.

Fig. 32. Abdominal sternites of normal male.

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# SEX-INTERGRADES IN FŒTAL PIGS.

## (Preliminary Report.)

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The hormone theory as an explanation of the type of sex-intergrade known as the free-martin has hitherto been limited in its application to conditions in cattle. The theory permits however, general applicability, and all species that are able to duplicate the conditions which in bovine species invariably produce the free-martin, would be expected to give rise to similar anomalous forms (Lillie, '17).

The conditions essential for the production of the free-martin are the establishment of a common circulation between a hetero-sexual pair of individuals at an early stage in their embryonic development. Thus certain sex-hormones liberated into the embryonic blood stream are common to both individuals, and succeed in modifying the normal course of sexual growth. The fact that it is invariably the female structures which are affected, and that they are modified in the male direction, leads to the further assumption that the male hormones are more precocious in their development than those of the female, and consequently are able to exert their influence without any counteraction on the part of the female. This assumption receives considerable support from histological evidence in that it has been observed that the interstitial cells, the supposed seat of sex-hormones, develop earlier in the testis than in the ovary. In cattle, such cells first occur in testes of 3 cm. embryos, and in ovaries of 82 cm. females (Bascom, '23). A similar precocity of male interstitial cells has been stated to occur in the pig (Allen, '03).

Species which are typically uniparous and in which the placenta is of the diffuse type, offer the most favorable opportunities for the production of chorionic fusions and resulting complications.

In the pig, the two apices of the chorionic vesicle do not enter into formation of the placenta. The chorion is thin in these regions, being stretched by the extension of the allantois inside. These terminal regions are practically devoid of blood vessels, and remain throughout the whole period of gestation as thin necrotic areas which may closely adhere to, or become invaginated into similar regions of adjacent vesicles. These, in virtue of their necrotic structure, prevent actual fusions of chorionic tissues.

Chorionic fusions may be occasionally found in swine, however. Blastocysts may come to lie either side by side or end to end, and then fuse. In each case obliteration of the intervening wall results in the formation of a single chorionic cavity. Such fusions are not often followed by vascular anastomoses, for the vessels of the individual circulations do not often come into close proximity. They may frequently be seen completely reversing their direction upon approaching each other. The total free-martin conditions are consequently of comparatively rare occurrence in the pig.

Investigation into this situation was prompted by the discovery of a pair of heterosexual pig twins among some twinning material kindly supplied by Dr. Newman, University of Chicago.

I would like to express my thanks to Dr. Newman for kindly placing his material at my disposal, and also for helpful advice throughout this work. To Dr. R. F. Shaner, University of Alberta, I am also indebted for valuable criticism, and to Swift and Co. for coöperation during the collecting of material used for this work.

An examination this summer of approximately 400 uteri in the stage of mid-pregnancy yielded 7 cases of twinning. These, with 6 collected the previous summer, and 4 from the collection of Dr. Newman, make a total of 17, of which 8 are judged to be dizygotic in origin, 3 monozygotic, 4 undetermined, a pair of conjoined twins and a double-headed monster.

Seven instances of inter-embryonic vascular connections were observed. In most of these, the anastomoses were of side branches and therefore comparatively weak. In two cases the twins showed a marked difference in size, accompanied by injuries of the smaller individual. There was here, I take it,

an unequal sharing of the blood supply, one twin receiving an excess of blood and thereby robbing its co-twin of the full amount required for normal development. In one instance, the twins measured 30 mm. and 13 mm. respectively. The smaller individual had not commenced disintegration, but was a case of acardia with several morphological abnormalities.

In 4 cases, the establishment of vascular inter-communications was followed by abnormalities of the sex-equipment of one of the component twins. I now propose to describe these four cases.

*Case No. 1.*—(Figs. 1 and 3.) The twins in this case measured 65 mm. and were heterosexual as determined by the external genitalia. The normal length of the chorionic vesicle, and the position of the embryos in its center, indicate that fusion had taken place in a side-by-side manner. Fig. 1 is a semi-diagrammatic representation of the chorionic vesicle and its contents. In the foreground is the amnion enclosing the female embryo (*A*), behind which its allantois extends the complete length of the exocœlom. The constriction in the allantois to the right of the embryo is no doubt caused by pressure of the male embryo (*B*), which lies behind allantois *A* at this point. The allantois of *B* lies in the background, and also extends the complete length of the vesicle.

The distribution of the allantoic vessels is irregular. The greater portion of the placenta is vascularized by embryo *A*, whose anterior and posterior paired vessels are of equal strength and course the total length of the chorion. Those of *B* are unequal, the anterior pair being extremely weak and lost to view upon entering the placenta. The stronger posterior pair courses side by side with the corresponding pair from *A*, and in more than one place the branches of the two systems anastomose.

On dissection of the embryos, the reduced size of the female gonads was at once apparent. Actual measurements were 2.2 mm. in length compared with 4.4 mm. of the testes. A difference as great as this I have not observed in any normal individuals at the same stage of development. Histological examination showed structural modifications similar in most respects to those described for early free-martins. Figs. 5, 6 and 7 are camera

lucida drawings through the widest parts of gonads of the female twin, a normal female, and the male twin. The reduced size of the free-martin ovary is at once apparent, such inhibition of growth being, as in bovine forms, the initial stage of modifications brought about by the action of the male hormone. In addition to reduction in size, there is also inhibition of characteristic ovarian differentiation. In the male, the germinal epithelium is reduced to a single layer of cells, and is separated from the primary sex cords by a well-differentiated layer of fibrous connective tissue. The rete occupies a central position, and radiating from it are the primary sex cords. In the normal female, the germinal epithelium has undergone extensive proliferation, and now forms a thick cortical zone of secondary cords. The primary albuginea lying between the medullary and the secondary cords is embryonic in type, and lacks the character of the male structure. The medullary portion of the ovary is occupied by the rete, and a few remaining medullary cords. In the free-martin gonad the cortical zone of secondary cords is practically absent. These cords commence their proliferation from the germinal epithelium in 25 mm. embryos (Allen, '03). Consequently their absence in a 65 mm. individual indicates decided inhibition of female differentiation. The rete is conspicuously large, but most of the medullary cords have undergone degeneration, and are present as an unorganized mass of cells. The cortical region consists largely of connective tissue, not so well differentiated as in the male, but forming, nevertheless, a definite layer beneath the epithelium. The germinal epithelium has for the most part been reduced to a single layer of cells, but in a few places there is what I take to be the initial stages of sex cord formation. A few clumps of cells appear to have been cut off from the epithelium, and are wandering in the albuginea where they are apparently undergoing degeneration and absorption.

Differentiation of the genital ducts has barely commenced in embryos of this age, therefore irregularities have not occurred to any great extent. The narrowing of the Wolffian duct which takes place in normal females of this age, had not occurred in the free-martin.

*Case No. 2.*—This case is practically a repetition of the one just described. The twins measured 45 mm. and were heterosexual. Their position in the chorionic vesicle and the orientation of their membranes was similar to case No. 1. Anastomoses of veins occurred in places marked in Fig. 4. The same abnormality of the female sex-equipment was present here also, but this being a slightly younger stage, the medullary cords were more conspicuous, and still retained their cord-like structure. The cortical zone of secondary cords was again represented by a few clumps of degenerating cells in the tunica albuginea.

*Case No. 3.*—(Fig. 2.) This case differed from the previous in that the monochorionic condition had arisen from end-to-end fusion of blastocysts. This was evident both from the double length of the chorionic vesicle, and from the orientation of the embryos and their membranes. The zone at which fusion had occurred was indicated externally by a mere thinning of the placenta which stretched uninterruptedly across this zone, without leaving any signs of imperfect fusion in the form of persistent necrotic areas. The blood vessels of the two circulations here came into contact and inter-communicated by means of distinct venous anastomoses.

The fœtuses measured 9 cm. Modifications of the female sex-equipment have resulted in small testis-like gonads completely devoid of any trace of secondary sex cords (Fig. 8). In no place is the germinal epithelium more than one cell in thickness, indicating that secondary sex-cords, if present earlier, have by now been completely re-absorbed. The underlying tunica albuginea is typically male in structure, even to the extent of penetration of blood vessels. The medullary region is conspicuous and contains numerous sex cells arranged in clumps. These are being surrounded by connective tissue growing in from the tunica, and have thereby the appearance of early seminiferous tubules.

The Wolffian ducts were large throughout their entire course, not showing the normal degeneration at their anterior portions, and the Müllerian ducts had followed the male course of growth in becoming discontinuous in the region immediately posterior to the gonads.

*Case No. 4.*—(Figs. 11 and 12.) This case presents altogether a different condition from the three previous. A free-martin is a zygotic female co-twin to a male. The twins in this case were two males and measured 6.3 cm. and 7.8 cm. respectively. Their position and orientation in the chorionic vesicle was identical with that of cases Nos. 1 and 2. The vascular anastomosis was stronger than in any other case and was doubtlessly responsible for the difference in rate of growth of the two embryos. Consequent upon an excess of the blood supply being received by one twin, the less fortunate individual was deprived of the full amount necessary for complete growth and normal development.

The external genitalia of both individuals was typically male in structure. The scrotal sacs, which were plainly marked in the larger male, however, were indiscernible in the smaller.

Examination of the internal genital systems, showed the larger individual to be quite normal. The testes, measuring 4.4 mm. by 2.2 mm. had not commenced their descent. The mesonephroi were large and without any signs of shrinkage. Fig. 9, representing a section through the urogenital folds immediately posterior to their fusion, shows the large size of the Wolffian ducts. The Müllerian ducts persist in this region, but farther anteriorly become intermittent.

The smaller individual presented the condition illustrated in Fig. 12. The testes measured 3.9 mm. in length and 1.2 mm. in width. Descent was in progress, the left having out-distanced the right by more than its own length. Degeneration of the mesonephroi which occurs normally in pigs of 10 cm. or more, is here precocious and irregular. All that is left of the left mesonephros is a narrow ridge of tissue accompanying the genital ducts. The right has retained its normal size at its anterior end and is extremely irregular in its posterior region.

On section, the testes proved to be normal in structure. Compared with those of the co-twin, they were more primitive however, being only one half the diameter, and possessing a fewer number of tubules. The tubules contained fewer sex-cells, and a great abundance of inter-tubular material was present.

Fig. 10, representing a section through the genital ducts at a

region corresponding to that in Fig. 9, indicates the difference in size of the two Wolffian ducts. The left duct is approximately as small as its accompanying Müllerian duct, and even that on the right does not approach the normal size. The Müllerian ducts could be traced throughout their whole length, and the left was especially large. Degeneration does not occur in the region indicated in diagram until a later date, hence no irregularities in size are evident here.

## Discussion.

A considerable number of sex-intergrades in adult swine have been described during the last few years. The free-martin condition as a possible explanation has been discussed and abandoned partly on the grounds that chorionic fusions with their complications have not been described in the pig. There is no doubt that the first three cases described in this paper would have developed into typical sterile free-martins such as occur in cattle. Their gonads might be structural testes, or, in consideration of the fact that a certain amount of secondary sex-cord proliferation takes place, they might possibly be ovo-testes. The temporary appearance of the Pflüger cords and their consequent degeneration, as described in the first two cases, is coincident with the development of the interstitial cells in the female gonad. It has been pointed out by Allen ('03), that these cells first appear in scanty quantities in the ovary, at the same time as they appear in the testis in large numbers, but that they subsequently disappear in the ovary not to reappear until a much later period of development. With their disappearance the male hormone is able to exert its influence without any counteraction on the part of the female, and degeneration of the few female sex-cords results.

Case No. 4 presents a different proposition. Similar abnormalities in adult swine have been explained by Crew ('23) on the hypothesis of quantitative sex-differentiating stimulus. A minimum amount of stimulus is required at certain critical moments during development for complete organization of one sex and suppression of the other. A retardation in the production of such stimulus, or an insufficiency in quantity thereof, results in incomplete organization of the one sex and suppression of the other. In the case under discussion, insufficiency of the required

stimulus may be supposed to have been caused by the loss of blood following the establishment of a common circulation between the twins. This took place after differentiation of the external genitalia, and consequently these are normal in structure. Later developments, including complete differentiation of the genital ducts, degeneration of the mesonephroi, growth and descent of the testes, have been affected, and these therefore show evidence of irregular and uncontrolled growth.

These are only suggestions, however. More material is required to clear up these doubtful points, and it is hoped that further investigation will determine more definitely the extent to which intra-uterine conditions are responsible for the production of sexual abnormalities in the pig.

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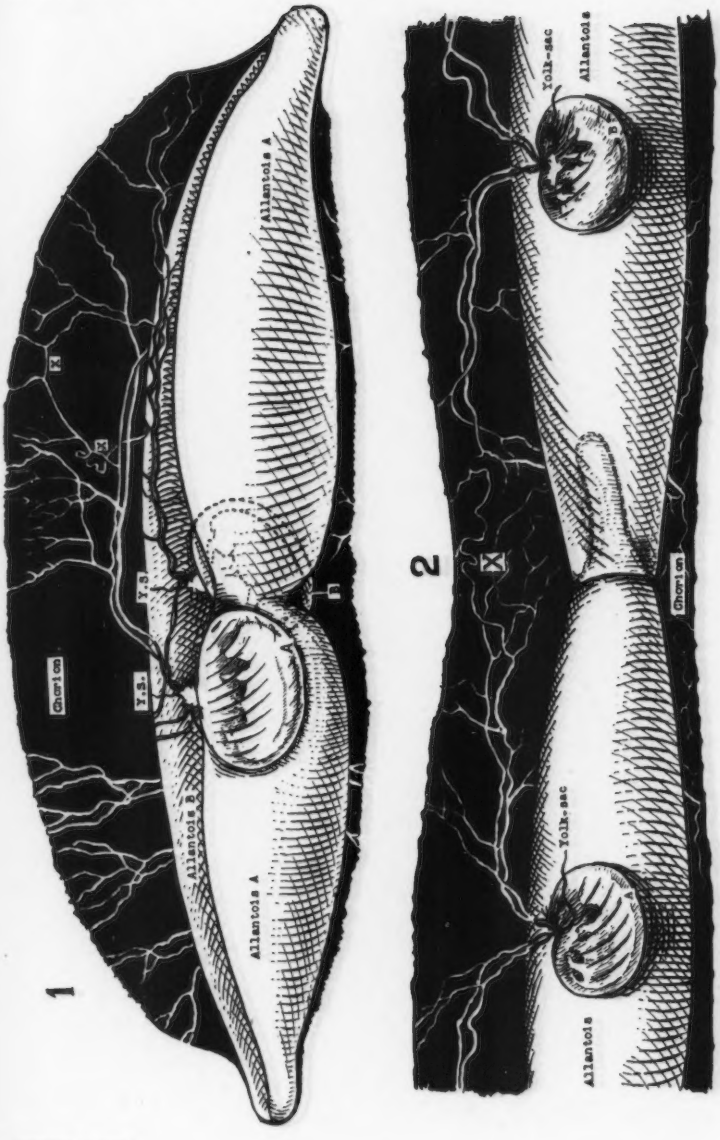
PLATE I.

FIG. 1. Case No. 1. *A.*, amnion enclosing female twin; *B.*, amnion enclosing male twin; *Y.S.*, yolk-sac; *X.*, anastomoses of vessels.

FIG. 2. Case No. 3. Terms as in preceding figure.

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## PLATE II.

FIG. 3. Case No. 1. Embryonic membranes removed to show vascular systems.

FIG. 4. Case No. 2.

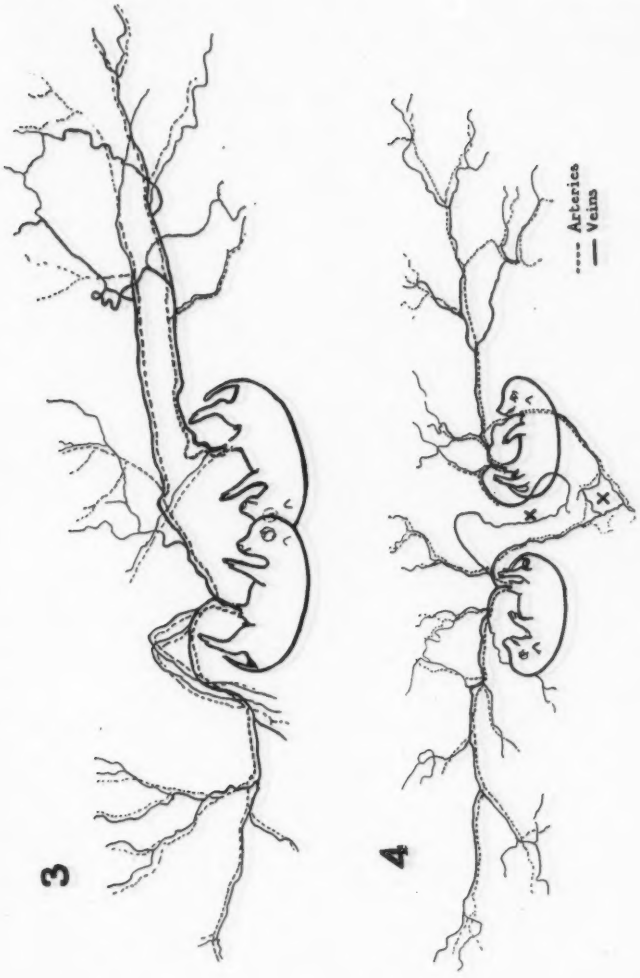


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PLATE III.

FIG. 5. Modified ovary of free-martin, case No. 1.

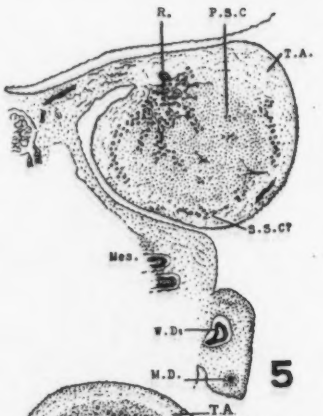
FIG. 6. Ovary of normal 65 mm. embryo.

FIG. 7. Testis of male twin case No. 1.

FIG. 8. Modified ovary of free-martin No. 3.

FIG. 9. Genital ducts of normal male twin, case No. 4.

FIG. 10. Genital ducts of abnormal male twin, case No. 4. *Mes.*, mesonephros; *M.D.*, mullerian duct; *P.S.C.*, primary sex cords; *R.*, rete; *R.O.*, rete ovarii; *R.T.*, rete testis; *S.S.C.*, secondary sex cords; *T.A.*, tunica albuginea; *W.D.*, wolffian duct.


R.
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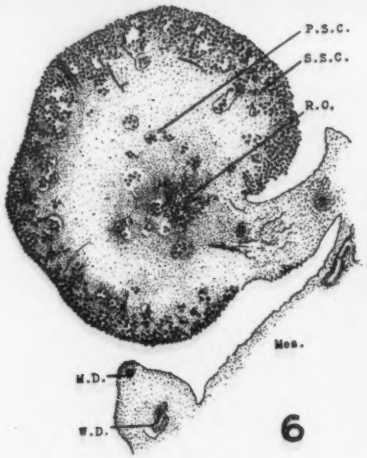
T.A.
S.S.C?
Mes.
W.D.
M.D.

5


P.S.C.
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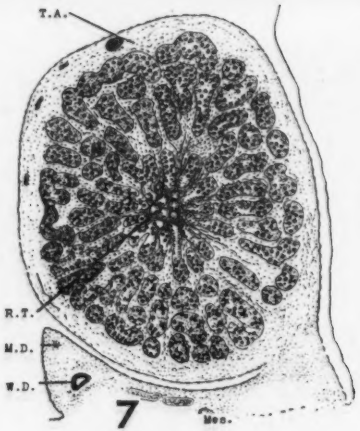
W.D.

6


T.A.
R.T.
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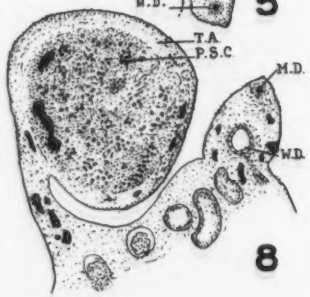
Mes.

7


T.A
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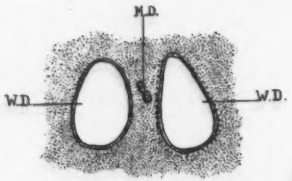
M.D.
W.D.
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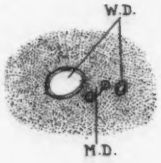
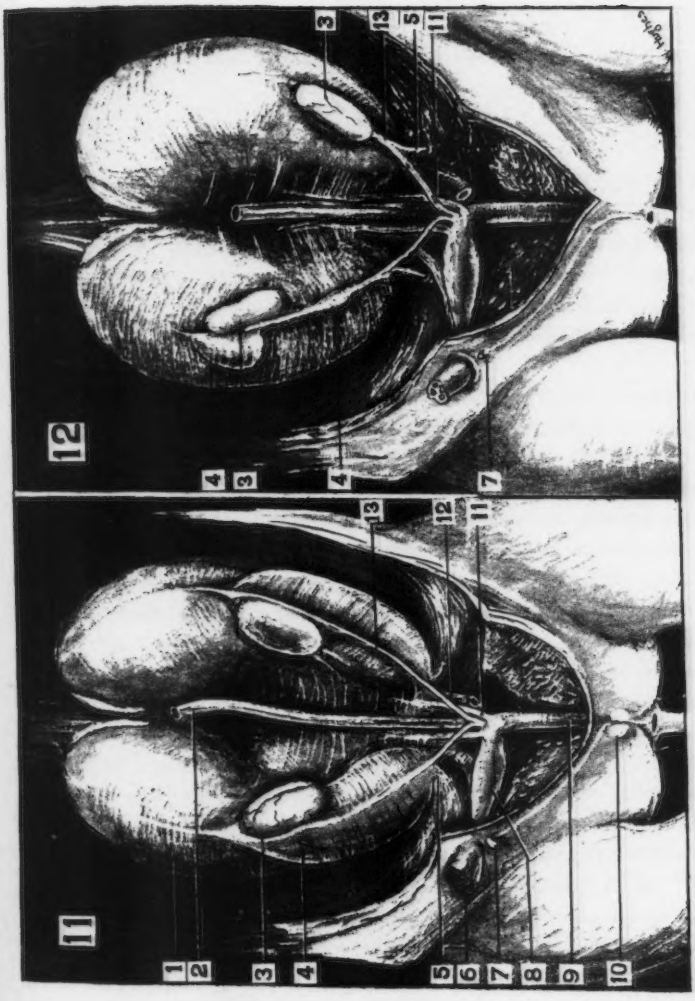

W.D.
M.D.

10

PLATE IV.

FIGS. 11 AND 12. Normal and abnormal twins of case No. 4. 1, kidney; 2, gut; 3, testis; 4, mesonephros; 5, inguinal fold; 6, umbilical cord; 7, penis; 8, allantois; 9, prostatic urethra; 10, scrotal sacs; 11, ureter; 12, allantoic artery; 13, genital ducts.




WINIFRED HUGHES.

# SEX-REVERSAL IN PARABIOTIC TWINS OF THE AMERICAN WOOD-FROG.[1]

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On the basis of his work on the free-martin F. R. Lillie (1917) expressed the view, "that sex-determination in mammals is not irreversible predestination, and that with known methods and principles of physiology we can investigate the possible range of reversibility." Numerous investigators followed this suggestion in an effort to determine the nature of the sex-differentiating factors.

Parabiosis experiments which were first performed in rats by Morpurgo in 1908, were carried out by Matsuyama (1919), Yatsu (1921) and Pfeiffer and Zacherl (1926). These authors obtained almost identical results in so far as there was no sex-change observed. Parabiosis here does not even prevent copulation or suppress fertility in either sex. Yatsu describes some pathological changes taking place in the ovaries and the uteri of the female, while the male co-twin is never impaired in the least by the female. But as the castrated male exerts the same influence as the normal one, it seems improbable, that we have to do in this case with a specific action of male differentiating factors.

The experiments were then extended upon birds and amphibians, in which the embryos are accessible for the experiment long before their sexual differentiation. Minoura (1921) first reported some intersexual features in the female chick embryos, caused by implanted pieces of the testes of adult cocks. But Greenwood (1925), when repeating that experiment did not find such influences. Recently Willier (1925) grafted embryonic

[1] The experiments were carried out in the Osborn Zoölogical Laboratory, Yale University. The microscopical study of the material was continued at the Marine Biological Laboratory, Woods Hole, Mass. The writer takes this opportunity of expressing his obligation to these institutions and feels also very much indebted to the International Education Board for the grant of a fellowship.

chick gonads, shortly before or after their sexual differentiation, into the chorio-allantoic membrane of nine-day-old host embryos. The material was preserved nine days after the operation. In no case of heterosexual combinations was there found a specific modification in the process of sex-differentiation of the graft, nor any trace of sex-reversal in the gonads of the host embryo.

Briefly, we can say that the experiments with mammals and birds failed to show a definite indication of a tendency towards a physiological sex-reversal.

On the other hand Burns (1925) reported a very successful experiment on the urodele *Amblystoma*. He joined young embryos during the tail-bud stage in parabiosis. Instead of the expected chance combinations of the sexes, requiring a ratio of 1♂♂ : 1♂♀ : 1♀♂ : 1♀♀, he obtained exclusively one-sexed pairs.[1] Burns is inclined to interpret the numerical result of 44♂♂ + 36♀♀ as a 1 : 1 ratio and infers, that in about one half of the original ♂♀ and ♀♂ combinations the males changed into females, while in the other half, the females changed into males. He claims, that "there is no prepotency, which constantly favors a given sex." But he did not happen to see any developmental stage of the supposed sex-transformation. He finds the pairs to be one-sexed already in the very earliest phases of sex-differentiation. Therefore the essential part of the process remains obscure. Burns' experiments, although successful in showing that a transformation of sex occurs, did not solve the problem of how it occurs.

In the spring of 1926 the writer started new experiments in which he joined young frog embryos in parabiosis. The operation was performed shortly after the closure of the medullary tube; that is, at an age of approximately 50 to 70 hours, when the eggs were kept in the laboratory at room temperature (18°–20° C.). In normal controls the first sex-differentiation takes place during the third week. In the parabiotic twins it is often somewhat delayed. The twins were preserved at intervals during the larval period and the stage of metamorphosis (Fig. 1).

The material belonged to four different species, but in the following we consider only the experiments with *Rana sylvatica*, the American wood-frog.

[1] ♂♀ means male on the left, female of the right side; and vice versa.

Controls were reared under identical laboratory conditions. Sex-differentiation was in full course by the 22d day, in a lot of larvæ, preserved at this time. The animals metamorphosed between the 44th and the 59th day. The species proved to

FIG. 1. Parabiotic twins TS1. Newly metamorphosed. The left side animal with the pigmented ventral surface is an European *Temporaria*, the right side animal is an American *Sylvatica*.

belong to the differentiated sex type. Among these 196 controls were 100 females and 96 males—a very close approximation to a 1 : 1 ratio (expectation calling for 98 : 98 with a probable error of $\pm$ 4.7).

We should therefore expect, that by chance combination, the 56 twins considered in this paper would consist of the following zygotic groups—14♂♂ + 14♂♀ + 14♀♂ + 14♀♀ (the probable error being $\pm$ 2.2). Preliminary observations on a part of this material seemed to indicate that in the heterogeneous combinations the sex of one animal is not influenced by that of the other, and a statement to this effect was made in an evening lecture at the Woods Hole Laboratory this summer.

There are indeed a considerable number of cases like No. 44, in which one animal possesses normal testes, while the other has ovaries, which do not show any apparent deviation from the

FIG. 2. Parabiotic twins SS44. (Both are *Rana sylvatica*.) Left larva, 34 mm. total length. 68 days after the operation. Male. Transverse section through one of the testes. The germ cells (primary spermatogonia) are in the sex cords. × 444.

typical female conditions (compare Figs. 2 and 3). Later investigations, however, showed, that some of the females twinned with males underwent sex-reversal. In three such cases large parts of the gonads show a nearly typical testicular structure (Fig. 4), while other parts are ovarial or show progressive stages in the transformation process. In seven more cases the gonads are in the first stages of that transformation (Fig. 5). Such ovaries are, without exception, very slender. The same thing is observed in some cases, in which the reorganization has not yet begun. Obviously an ***inhibition in ovarial growth*** is the first visible effect exerted by the male co-twin.

From the fact, that the reverse combination of a female with a sex-changing co-twin was never found, it becomes evident, that *the male sex dominates the female one.*

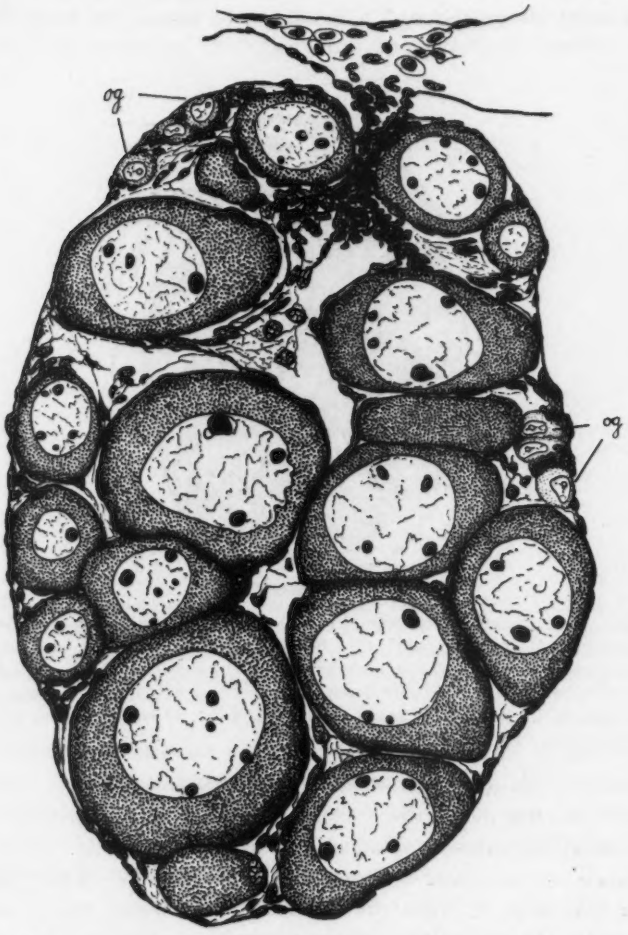


FIG. 3. Same pair SS44. Right larva, same length as left one. Female. Transverse section through one of the ovaries. The germ cells (primary oögonia and auxocytes) are in the peripheral germinal epithelium. *og.*, primary oögonia. × 444.

With regard to the last two points, the above statements are in full agreement with Lillie's observations on free-martins. However, while the latter never effect a complete sex-change, and do not produce male germ cells, the changing frog twins apparently are on the way to become true males. In frogs, ex-

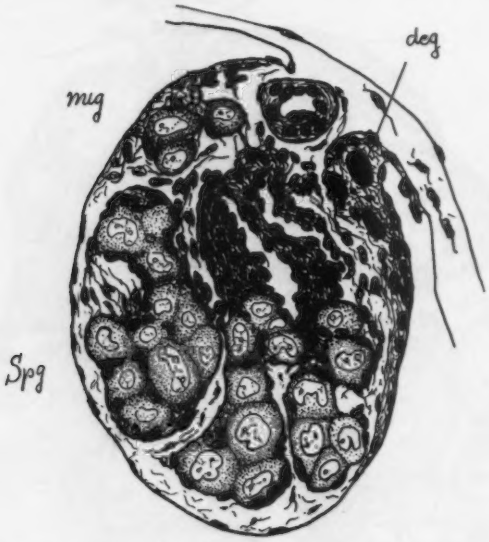


FIG. 4. Parabiotic twins SS40 (*R. sylvatica*). Right animal shortly after metamorphosis. 68 days after the operation. Female changing into male. Transverse section through the posterior part of the gonad which shows nearly typical male conditions. Highly developed sex cords, relatively small number of spermatogonia. Rests of the former germinal epithelium at *mig.* (migrating germ cells) and *deg.* (degenerating oögonia). × 444.

hibiting a transformation of sex, the undifferentiated germ-cells, which in the ovary are exclusively found in the peripheral germinal epithelium (so-called primary oögonia, Fig. 3, *og.*) migrate to the center of the gonad (Fig. 5, *mig.*). They join the sex-cords, to form the characteristic lobules, which are known to be the anlagen of the seminiferous tubules (Fig. 4), (Witschi, 1914; Swingle, 1926). From this stage they are therefore to be considered as *spermatogonia* (Fig. 4, *spg.*).

The described facts are in many respect in apparent contrast

to those reported by Burns (1925) in *Amblystoma*. This author did not find that the male sex predominates or even that sex-transformation decreases the growth of the involved gonads. Furthermore, he supposes that the change of sex takes place before the time of sex-differentiation. Let us consider the latter point with respect of the present experiments with *R. sylvatica*.

FIG. 5. Parabiotic twins SS41 (*R. sylvatica*). Left larva, 38 mm. total length. 68 days after operation. Female changing into male. Transverse section through one of the gonads, showing inhibition of growth, disintegration of the germinal epithelium and formation of the testis in the center of the gonad. *mig.*, germ cells migrating into the sex cords. × 444.

Supposing there were such an early sex-reversal in the frog twins, then the number of one-sexed pairs ought to be increased at the expense of the two-sexed ones. However, the statistical evidence is against such an assumption. There were found the following combinations:

♂♂—16
♂♀—17 (seven of the females undergoing sex-reversal)
♀♂—10 (four of the females undergoing sex-reversal)
♀♀—13

The result of 29 one-sexed and 27 two-sexed pairs is quite close to expectation ($28 \pm 2.5$). Among the total of 112 individuals we find 59 ♂ and 53 ♀ (the latter group including the sex-changing animals). The deviation from the mean is less than the single P.E. It can be considered, therefore, as well established, that *there was no sex transformation before the time of sex-differentiation.*

In discussing the fact of the dominance of the male sex in the free-martin, Lillie is inclined to consider the time-factor as an essential one. The observations of his students (Chapin, 1917 and Bascom, 1923), relating to the earlier development of interstitial cells in the testes and the later differentiation of the ovaries, seem to favor the theory of dominance of the male twin by earlier hormone production. Lillie admits, however, that other causes may also exist. In the case of our frog twins it is evident that the time-factor has not the suggested significance. The male-differentiating factor reaches the female gonads after the ovaries have acquired a relatively advanced stage of development. This means, that the female-differentiating factor is already in full action. In the resulting competition of both, the male-differentiator acts first as an inhibitor and later on as a suppressor of the female one. This is in strict agreement with the conclusions derived from the author's observations on hermaphroditism (1914; 1921). I think, that there can no longer be any doubt about *the antagonistic character* of these two factors.

Lillie (1923) and Bissonnette (1924) report on some earlier stages of the free-martins with rather highly developed ovarial structures. It seems not improbable that the female co-twins started with a typical female sex-differentiation. Such cases would lead to the same conclusions as the parabiotic frog twins. The assumption, that only the male is producing hormones in an early time, would apparently not be sufficient to explain the suppression of the active ovarial development. There seems to be no escape from the postulated sexual antagonism.

Some years ago Goldschmidt (1920) put forward the idea, that the sex-determining genes act like enzymes, stimulating the production of sex differentiating hormones. The term

"hormones" taken in its proper sense, would indicate, that the sex-differentiators circulate in the blood and are spread throughout the entire body. The writer's previous studies on sex-differentiation in the frog seem to point in a somewhat different direction. In hermaphrodites the eggs are formed exclusively in the germinal epithelium, while the spermatogonia develop in the sex-cords. At the beginning of the sex-reversal from female to male those germ-cells situated closest to the sex-cords are the first to show characteristic changes and to migrate into the sex-cords. If we consider the behavior of the germ-cells as an indication of, or a reaction to, the nature of their environment, then we must attribute male differentiating properties to the sex-cords. On the other hand, from the fact that the germ-cells which were not attracted by the sex-cords, but which remained in the germinal epithelium, become eggs, it becomes evident that this epithelium includes female-differentiating properties. From such facts, which furthermore are supported by experimental data, I proposed the *theory of localized sex-differentiators* ("lokalisierte Innenfaktoren," 1914). These probably are comparable to Spemann's "Organisatoren."

The fact of independent sex-differentiation in genetically two-sexed twins favors again that localization theory. Regarding the secondary sex-reversal of the female part, the coöperation of hormones is not improbable. We may assume, either that part of the active substance elaborated in the sex-cords is carried away by the blood-stream, or that the differentiated testes produce a hormone, which is different from the substance inducing the first sex-differentiation. Yet, it is to be kept in mind, that—at least in the frogs—there is no definite proof, that the testes of the male twin act by way of the blood-stream. This question has to be discussed in connection with a detailed study of the homoplastic and heteroplastic twins, which will be published at a later date.

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